

# PETRIA

## Giornale di Patologia delle Piante

22<sup>nd</sup> International Conference

on

### **Virus and Other Graft Transmissible Diseases of Fruit Crops**



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Marina Barba, Francesco Faggioli, Vincenza Ilardi,  
Graziella Pasquini, Luca Ferretti



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Rome, June 3-8, 2012

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Participants at the 22<sup>nd</sup> ICVF



## PREFACE

*Dear Colleagues,*

*We are very happy and honored to welcome you for the second time in 18 years to Rome for the 22<sup>nd</sup> International Conference on Viruses and Other Graft Transmissible Diseases Fruit Crops. Some of you (the 'youngest' ones!) remember that in 1994 the Plant Pathology Research Institute hosted the 16<sup>th</sup> Symposium on Fruit Tree Virus Diseases and the 7<sup>th</sup> Symposium on Small Fruit Virus Diseases. The meeting was a great success and we hope to offer to you the same warm hospitality as we did 18 years ago!*

*More than 150 participants from different countries where fruit crops are economically important are here to present their most significant scientific results obtained over the last three years.*

*In addition to the paper and poster presentations, we have organized a round table discussion on the 'Diagnosis of relevant pathogens: the role of National and International organizations within the framework of harmonization'. The round table, chaired by Ms Françoise Petter, EPPO assistant Director, will involve the participation of scientists actively involved in this specific field.*

*In addition, a workshop is planned to update all the conference participants on the European Certification of fruit trees and small fruit crops. It will include the active participation of experts from different countries who are involved in the establishment of legal, scientific and operative aspects aimed at improving the phytosanitary quality of fruit trees and small fruit propagative material and at reducing the spread of pests through the commercialization of infected germplasm.*

*On the last day of the conference, we will host a COST workshop on 'Phytoplasmas in fruit trees: multidisciplinary approaches toward disease management' which will offer all participants the opportunity to be informed on special aspects of this class of plant pathogens.*

*During the conference we will have the pleasure of listening to four lectures presented by researchers who have spent most of their scientific careers in improving and advancing our knowledge of viruses, viroids, phytoplasmas as well as detecting and identifying plant viruses and viroids. A big thanks goes to Professors Martelli, Flores, Oshima and Hadidi who kindly accepted to share their long experience on these specific topics with us.*

*We thank all of you for attending this second meeting held under the new title including viruses and other graft transmissible diseases of fruit trees and small fruit crops. We hope you enjoy the meeting and the beauty of the eternal city, Roma!*

*Marina Barba*

*Convener of the Conference*

*and*

*Member of the Organizing Committee*



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## ADVANCES IN THE KNOWLEDGE OF SOME FRUIT TREE VIRUSES

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Two studies to identify the plant viruses that rank among the ‘top ten’ in the world in terms of their economical and scientific importance, carried out following the initiative of the late Dr. R.G. Milne (1988) and the journal ‘*Molecular Plant Pathology*’ (2011), included *Plum pox virus* (PPV) and *Apple chlorotic leafspot virus* (ACLSV) in the list. The properties of both viruses are reviewed taking into account recent developments in their knowledge. The agent of fig mosaic is a virus (FMV) with enveloped particles and a multipartite single-stranded negative-sense RNA genome. FMV is transmitted by eriophyid mites, the same as six similar viruses which are approved (FMV and *European mountain ash ringspot virus*) species or potentially putative members in the unassigned genus *Emaravirus*. Contrary to previous reports, the FMV genome was recently found to consist of six (-)RNA fragments, only three of which encode proteins with recognized functions. The family *Closteroviridae* comprises two fruit tree viruses [*Little cherry virus 2* (LChV-2), *Plum bark necrosis stem pitting-associated virus* (PBNSPaV)], which is classified as definitive species in the genus *Ampelovirus*, and one [*Little cherry virus 1* (LChV-1)], currently retained as an unassigned species to the family. Recently secured molecular information has prompted: (i) the revision of the genus *Ampelovirus* with the identification of two subgroups, one of which contains LChV-2 and PBNSPaV; (ii) the establishment of a putative novel genus comprising LChV-1, for which the name *Velarivirus* has been proposed.

## MOLECULAR ANALYSIS OF A 'NEW' *FOVEAVIRUS* ASSOCIATED WITH A FRUIT DEFORMING DISEASE OF APPLE

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### Abstract

There are several fruit deforming diseases of apple that appear to be virus related, but the specific causal agent(s) is unknown. Some of these include apple green crinkle, apple rough skin, and apple star crack disease. Susceptible apple cultivars are often severely affected by these diseases producing fruit that are deformed and unmarketable. A putative 'new' *foveavirus* was identified that may be associated with apple green crinkle disease (AGCaV). The genome of AGCaV consists of 9266 nucleotides, with an organization similar to *Apple stem pitting virus* (ASPV) the type species of the genus *Foveavirus*, family *Flexiviridae*. ORF1 of AGCaV encodes a replicase-complex polyprotein with a molecular mass of 247 kDa; ORFs 2, 3, and 4 (TGB proteins) are estimated at 25.1, 12.8, and 7.4 kDa, respectively; and ORF5 encodes the CP with an estimated molecular mass of 43.3 kDa. The virus has four non-coding regions (NCRs) that include a 5'-NCR (60 nts), a 3'-NCR (134 nts), and two intergenic (IG) NCRs - IG-NCR1 (69 nts) and IG-NCR2 (91 nts). AGCaV is closely related to ASPV, but appears to be a distinct *foveavirus*.

## Introduction

Apples (*Malus domestica*) are a very important fruit crop globally, and are cultivated in many countries (Stebbins and Aldwinckle, 1990). Apples are affected by a range of graft-transmissible diseases, some with the causal agent(s) identified, but many of unknown aetiology. Some important fruit deforming graft-transmissible diseases of apple of unknown aetiology include: apple rough skin, apple russet ring, apple star crack, and apple green crinkle disease (Hansen and Parish, 1990). Susceptible apple cultivars are often severely affected by these diseases, producing fruits that are deformed and unmarketable. These diseases are important because usually symptoms are restricted to the fruit, meaning that a farmer could incur considerable expense nurturing and cultivating the infected trees until they reach maturity, only to produce a crop of deformed fruit with little or no value.

The disease apple green crinkle was described early in the 20th century (1930s) as kikei-ta disease in Japan in 1935 (Sawamura, 1965), and in New Zealand in 1937 (Atkinson and Robbins, 1951). The disease was presumed to be caused by a virus, based on its graft transmissibility (Atkinson and Robbins, 1951; Sawamura, 1965). Symptoms of the disease appear only on the fruit of susceptible cultivars and include: depressed areas, dwarfed and severe fruit malformation, cracking, brownish-red spots, wart like swellings, some with rough brownish-red russetting, and symptoms may be unevenly distributed among fruit from different branches of the tree (Hansen and Parish, 1990; Nemeth, 1986; Sawamura, 1965). Severe tree decline is associated with some isolates of the disease, and temperature influences symptom development. Mean daily temperatures that stay above 12.5°C prevent symptom development in cultivars such as Granny Smith (Hansen and Parish, 1990).

Recently, a virus was detected in 'Aurora Golden Gala' growing in Kelowna, BC, Canada displaying severe symptoms of apple green crinkle disease. The complete sequence of the virus was determined. The polyvalent degenerate oligonucleotides (PDO) RT-PCR assay described by Foissac *et al.* (2005) was used to screen for the presence of the virus in a range of green crinkle affected apple trees from various sources. This new virus was found to be associated with green crinkle disease and has been designated *Apple green crinkle associated virus* (AGCaV).

## Materials and Methods

**Virus isolates:** Leaf samples from healthy 'Aurora Golden Gala' apple trees, and leaf and fruit samples from 'Aurora Golden Gala' trees showing apple green crinkle disease (AGCD) symptoms were obtained from Kelowna, BC, Canada. Samples were obtained also from apple trees in the virus collection at the CFIA's Sidney Laboratory, BC, Canada. These latter samples were indexed over several years. Their disease statuses are known, including accessions known to be affected by AGCD (Tab. 1). The *Apple green crinkle associated virus* (AGCaV) isolate targeted for sequencing is identified as isolate AGG-K10, and was detected in a 2010 accession of 'Aurora Golden Gala' apple showing severe symptoms of green crinkle disease.

**Tab. . 1.** Disease status of some apple accessions screened for the presence of the apple green crinkle associated virus (AGCaV)

CPH Accession	Disease Status <sup>1</sup> (Woody indexing etc)						Apple Variety
	AGC	ACLS	ASP	ASG	RW (woody indexing)	Other (woody indexing)	
1148-02-2P1	2+	+	+	+	-		Golden Del
1819-18-18B1	+	+	+	-	-		unknown
1907-01-1P2	+	+	+	+	-		Golden Del
1907-03-3P2	+	+	+	+	-		Golden Del
1907-04-4P1	+	+	+	+	-		Golden Del
1907-05-5P1	+	+	+	+	-		Golden Del
1148-09-9P1	-	+	+	+	-	Chat fruit	unknown
982-11-11P4	-	+	+	+	+	Flat limb	Courtland
70-08-8P6	-	+	-	-	+	Stem Decline	Red Delicious
1920-01-1A2	-	-	+	-	-		unknown
1920-08-8A1	-	-	+	-	-		Red Delicious
264-05-5P6	-	+	+	+	+	Stem Decline	Kinsei
2014-02A3	-	-	-	-	-		Healthy Aurora Golden Gala

<sup>1</sup>Disease status was determined by woody indexing and confirmed by ELISA and/or RT-PCR when available. Apple disease abbreviations used include: AGC = apple green crinkle; ACLS = apple chlorotic leaf spot; ASP = apple stem pitting; ASG = apple stem grooving; RW = rubbery wood. 2+ = positive; 3- = negative.

**Double stranded (ds) RNA extractions:** Approximately 20 g aliquots of leaves from healthy ‘Aurora Golden Gala’ apple trees, and from AGCD symptomatic trees infected with AGCaV isolate AGG-K10 were used for dsRNA extractions. Extractions were carried out essentially as described by Morris and Dodds (1979).

**Total RNA extractions:** Total RNA was extracted from all plant samples using Qiagen’s RNeasy Total RNA kit, and following the supplier’s (Qiagen, Chatsworth, CA) instructions.

**Cloning, sequencing, and sequence analysis:** Purified dsRNA was gel purified and denatured, then cDNA was generated using an oligo(d)T primer combined with random hexamers. Once initial virus-specific sequences at the 3’-terminus of the genome were obtained, AGCaV-specific reverse primers were designed and combined with random primers for genome sequence walking to the 5’-terminus. The 5’-terminus of the virus was determined using the 5’-RACE system (Invitrogen, Burlington, Ontario). Amplified fragments were gel purified using a MiniEute Gel Extraction kit

(Qiagen, Mississauga, Ontario), and the purified fragments were ligated into the pCR-TOPO vector and cloned using the TOPO TA Cloning kit (Invitrogen). Sequencing was carried out at the Nucleic Acid Protein Service Unit, UBC, Vancouver, BC, using an Applied Biosystems 3730 DNA Analyzer. Sequence analysis was performed using Clone Manager 9 Professional Edition (c) 1994-2010, Scientific and Educational Software, Cary, NC, USA. Phylogenetic analyses were carried out using Clustal X, v1.83 (Thompson 1997).

**Polyvalent Degenerate Oligonucleotides (PDO) RT-PCR and sequence analysis:** PDO RT-PCR was conducted as described by Foissac *et al.* (2005). The 362 bp products obtained were gel purified, cloned, and sequenced as described above.



Fig.1 - Genome organisation of the apple green crinkle associated virus (AGCaV), which is similar to that of *Apple stem pitting virus* (ASPV) the type member of the genus *Foveavirus*, family *Betaflexiviridae*. Abbreviations indicate: methyltransferase (M), cysteine protease (C), papain-like protease (P), helicase (Hel), RNA-dependent RNA polymerase (Pol), coat protein (CP), and open reading frame (ORF).

## Results

The complete genome of AGCaV was determined to consist of 9266 nucleotides, excluding a poly(A) tail at the 3' terminus of the virus genome. At least 3 independently derived clones, sequenced bi-directionally and covering overlapping regions, were used to assemble the virus genome. Five open reading frames (ORFs) and four non-coding regions (NCRs) were identified. The genome organization of AGCaV isolate AGG-K10 is similar to that of *Apple stem pitting virus* (ASPV), the type member of the genus (Fig. 1). ORF1 encodes the replicase-complex polyprotein, Mr 247 kDa; ORFs 2, 3, and 4 encode the triple gene block (TGB) proteins with sizes Mr 25.1 kDa, 12.8 kDa, and 7.4 kDa, respectively; and ORF 5 encodes the virus coat protein with Mr 43.3 kDa. The NCRs include a 5' NCR of 60 nts, a 3' NCR of 134 nts, and two intergenic (IG) NCRs (IG-NCR1 of size 69 nts between ORF1 and ORF2, and IG-NCR2 of size 91 nts between ORF4 and ORF5).

The following identities were observed between AGCaV isolate AGG-K10 and ASPV isolate PA66, accession number D21829.1 (aa sequence homology indicated in brackets): the complete genomes (nt) are 77% identical; ORF1, 78% (85%); ORF2, 77% (91%); ORF3, 82% (90%); ORF4, 83% (83%); and ORF5, 69% (72%).

Phylogenetic analysis conducted to determine the relationship of AGCaV to members of the genus *Foveavirus*, with complete genome sequences available, revealed a close but distinct relationship (supported by high bootstrap values) to a group of ASPV isolates that include the type isolate ASPV PA66 (Fig. 2). There are putative isolates of ASPV (e.g. KL9, KL1, and IF38) that appear also to be distantly related (supported by high bootstrap values) to ASPV PA66.

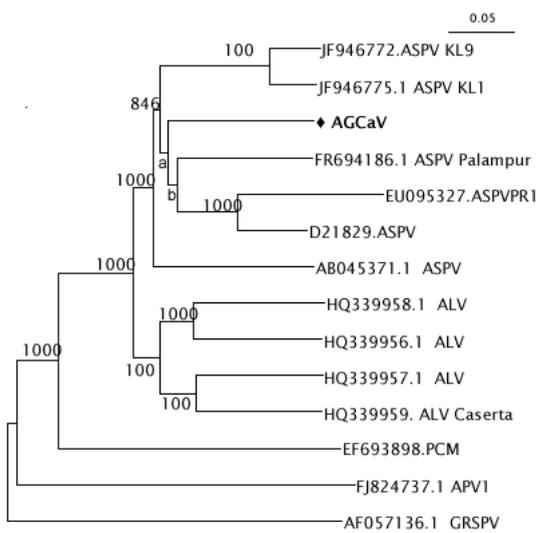


Fig. 2 - Phylogenetic analysis of the complete genome sequence showing the relationship of apple green crinkle associated virus (AGCaV) to viruses in the genus Foveavirus with their complete sequence available. Viruses include Apple stem pitting virus (ASPV), Apricot latent virus (ALV), Peach chlorotic mottle virus (PCMV), Asian prunus virus (APV), and Grapevine rupestris stem pitting virus (GRSPV). The tree was created using the N-J Tree option of Clustal X (Thompson et al., 1997). Bootstrap values out of 1000 replicates are shown. In the figure the letters a and b indicate bootstrap values of 910 and 730, respectively. The scale bar indicates the number of substitutions per residue.

Using PDO RT-PCR with cloning and sequencing of the 362 bp amplicons produced, phylogenetic analyses based on this fragment confirmed the presence of AGCaV in AGCD affected plants (Tab. 1). AGCaV was identified in all the green crinkle affected plants listed (data not shown).

## Discussion

AGCD symptoms were first observed over 70 years ago. The graft transmissible nature of the disease was determined shortly thereafter (Sawamura, 1965; Atkinson and Robbins, 1951), and a viral causal agent was proposed. The causal agent of AGCD has not yet been identified; consequently woody indexing is the only method available for monitoring for this disease. Woody indexing, however, is time-consuming, and results are influenced by weather conditions (Stouffer and Fridlund, 1989). Identification of the causal agent would facilitate the development of molecular assays for rapid and reliable detection.

In this study, an ASPV variant or putative new *Foveavirus* was associated with AGCD, and identified as AGCaV. In phylogenetic analyses based on complete genome sequences, AGCaV groups distinctly from the well characterized type ASPV isolate PA66 (accession # D21829). These differences are supported by high bootstrap values. It is distinct also from other foveaviruses included in the analysis, including isolates described as ASPV but that may meet the criteria for distinct virus species. Based on the International Committee on Taxonomy of Viruses (ICTV) criteria for species demarcation in the genus *Foveavirus* (Fauquet *et al.*, 2005), AGCaV may represent a new species. The nt and aa sequences of the CP of AGCaV are 69% and 72% identical, respectively, when compared to

the corresponding sequences of the definitive ASPV isolate PA66. This is within in the range recommended for the demarcation of distinct species within this genus.

AGCaV was detected in all apple isolates confirmed to be affected by AGCD. Interestingly, Apple chlorotic leaf spot disease (caused by *Apple chlorotic leaf spot virus*, ACLSV), apple stem pitting disease (caused by *Apple stem pitting virus*, ASPV), and Apple stem grooving disease (caused by *Apple stem grooving virus*, ASGV) often together in plants affected by AGCD. However, there are apple accessions that are infected with all three viruses (ACLSV, ASGV, ASPV), but display no symptoms of AGCD (Tab. 1). Also, at least in one case AGCD was observed in the absence of ASGV infection. These three viruses do not appear to cause AGCD normally. There may be a causal agent that is as yet unidentified, or perhaps a unique virus variant that causes symptoms of AGCD. AGCaV may be a variant of ASPV, or a new *foveavirus* associated with AGCD and is a good candidate for the causal agent of this disease. Studies are ongoing to further characterize AGCaV, and determine its distribution.

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## GENOMIC CONFORMATION OF *APPLE MOSAIC VIRUS* TURKISH ISOLATES COAT PROTEIN GENE REGIONS

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*Apple mosaic virus* (ApMV) is the most important virus infection of apple and hazelnut orchards in Turkey. The disease is destructive and epidemic on apple and hazelnut plantations, causing considerable growth reduction and yield loss. Major symptoms are yellowish white evident systemic mosaic on apple leaves and severe systemic mosaic, ring spots and oak leaf pattern on hazelnut. Hazelnut isolates were collected from the Black Sea region and the apple isolates from the major apple growing provinces and all were identified serologically and molecularly using the primers of genes encoding the coat protein region of ApMV. Coat protein gene targets of Turkish isolates were sequenced and to date, 15 data (7 apple and 8 hazelnut) were present in the NCBI databank. Single nucleotide polymorphisms were detected in Turkish apple mosaic isolates. In phylogenetic analysis performed with isolates selected from different locations and from different hosts throughout the world, Turkish apple and hazelnut isolates were detected in different regions and hazelnut targets were different at the rate of 50% compared to the other isolates selected from the NCBI Genbank. No significant differences were present in the aminoacide sequences.

**MOLECULAR CHARACTERIZATION OF *TOMATO RINGSPOT VIRUS* (ToRSV) STRAIN RESPONSIBLE FOR 'PRUNE BROWNLINER' DISEASE**

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*Tomato ringspot virus* (ToRSV) mainly infects different species of ornamental trees, fruit trees, and shrubs. ToRSV causes serious diseases in stone fruit, such as 'pruner brownline', whose main symptoms are a dark brown line at the graft union with pits and grooves in the woody tissue. In Chile this disease has been observed in plum cv. D' Agen grafted on Myrobalan rootstock. ToRSV was detected by RT-PCR using specific primer pairs designed by Griesbach (D1r: 5'-TCCGTCCAATCACGCGAATA-3'; U1f: 5'-GACGAAGTTATCAATGGCAGC-3'; 449bp). The viral isolate was transmitted and maintained in *Gomphrena globosa* L. Identification was confirmed by RT-PCR using a primer pair (ToRSV2r: 5'-CTCACGTAAATGTATGGTTCC-3'; ToRSV2f: 5'-GACTGGAGGTTTGAGTGGC-3') that amplifies a fragment of 330bp in the gene encoding for the coat protein (CP). Primers were designed for amplification of the entire gene encoding the CP of the virus (1687bp). The amplicons obtained were purified and cloned. Putative recombinant clones were analysed by colony-PCR using primers to vector sequences flanking the polylinker. Amplicons obtained from three colonies per cloned fragment were sequenced in both directions. Sequence analysis was carried out using the partial (330bp) and complete (1687bp) CP sequences of the Chilean isolate (ToRSV Ch Gg). In both phylogenetic trees Ch Gg ToRSV was closely correlated to one isolate from raspberry whose origin is the USA, and is distant from those that induce 'mosaic yellow peach bud disease' (PYBM) and other isolates more commonly found in raspberries. This is the first molecular characterization of the entire gene encoding the CP of a ToRSV isolate causing 'pruner brownline' disease.

## INCIDENCE OF VIRAL DISEASES ON PEAR PLANTS AND THE MOLECULAR CHARACTERISTICS OF THREE PEAR VIRUSES IN CHINA

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### Abstract

During 2001-2011, field surveys were carried out to evaluate the incidence of viral diseases on pear plants grown in China. The most frequently found viral disease-like symptoms were chlorotic leaf spot, leaf ring spot, leaf vein yellowing and mosaic. A few cultivars showed serious fruit malformation with stone-pitting like symptoms. A total of 410 pear trees from 18 orchards in nine provinces were tested by RT-PCR for the presence of *Apple chlorotic leaf spot virus* (ACLSV), *Apple stem grooving virus* (ASGV), *Apple stem pitting virus* (ASPV) and a viroid (*Apple scar skin viroid*, ASSVd). The infection rates of ASGV, ACLSV, ASPV and ASSVd were 70%, 57.4%, 43.2% and 14.5%, respectively, and over 95% tested pear varieties were infected by one or more viruses. The complete CP and TGB genes sequenced from 31 and 52 ASPV pear isolates showed a highly divergence, and some CP genes had continuous nucleotide acid inserts or deletions. The partial and complete CP genes of 45 ASGV isolates and 22 ACLSV isolates from pear also showed high divergences. Those three viruses from pear grown in China had specific phylogenetic positions, which indicated that the molecular evolution of those viruses might be related to their host origins.

### Introduction

From 2001 to 2011 field surveys were carried out to evaluate the incidence of viral diseases in pear plants grown in China. The most frequently found viral disease like symptoms were chlorotic leaf spot, leaf ring spot, leaf vein yellowing and mosaic. At a few orchards, some trees re-grafted for variety replacement showed uneven reddish leaves, leaf curling and even tree decline. A few cultivars at some orchards showed serious fruit malformation with stone-pitting like symptoms. In order to understand the sanitary status of pear trees grown in China, a total of 410 pear trees

from 18 orchards in nine provinces were tested by RT-PCR for the presence of *Apple chlorotic leaf spot virus* (ACLSV), *Apple stem grooving virus* (ASGV), *Apple stem pitting virus* (ASPV) and a viroid (*Apple scar skin viroid*, ASSVd). The infection rates of ASGV, ACLSV, ASPV and ASSVd were 70%, 57.4%, 43.2% and 14.5%, respectively, and over 95% tested pear varieties were infected by one or more of those viruses. It was noticed that ACLSV and ASPV were usually associated with leaf ring spot and fruit stone-pitting.

Viruses are particularly problematic in vegetatively propagated fruit crops in which the viruses are transmitted by artificial propagation operations. In commercially cultivated pear trees, *Apple chlorotic leaf spot virus* (ACLSV, *Trichovirus*), *Apple stem pitting virus* (ASPV, *Foveavirus*) and *Apple stem grooving virus* (ASGV, *Capillivirus*) are widely occurring pathogens and can significantly reduce the fruit yield and quality (Cembali, 2003; Plese, 1975; Yanase, 1983). These viruses are generally asymptomatic in most pear cultivars. However, ACLSV causes leaf malformation and chlorotic rings or line patterns in some pear cultivars, and the severity of symptoms depends largely on the plant species and virus strains (Desvignes and Boyé, 1988; Mink, 1989; Rana, 2010). ASGV is also associated with pear black necrotic leaf spot (PBNLS) occurred in Korea (Shim, 2004). ASPV infection causes vein yellowing or red mottling of pear leaves (Schwarz and Jelkmann, 1998). The mixed infection of these viruses is very common in cultivated pear plants, and has caused top-working disease of apple and pear on susceptible rootstocks (Cembali, 2003; Desvignes and Boyé, 1988; Yanase, 1983). All these viruses are naturally disseminated by infected propagating materials.

The three viruses ACLSV, ASGV and ASPV have flexible filamentous particles and belong to the family *Betaflexiviridae* (Carstens, 2010), and are of the species of the genera *Trichovirus*, *Capillovirus* and *Foveavirus* (Carstens, 2010; Martelli *et al.*, 1997), respectively, and have single-stranded positive-sense genomic RNA. The genomic RNA of ACLSV consists of 7,474-7,555 nt excluding the poly (A) tail at its 3'-end, which contains three overlapping open reading frames (ORFs 1, 2 and 3) encoding a 216 kDa protein involved in replication, a 50 kDa movement protein (MP) and a 22 kDa CP, respectively. The genomic RNA of ASGV is about 6,500 nt and is 5' capped and 3' polyadenylated, consisting of two overlapping ORFs with ORF1 (6.3 kb) encoding a polyprotein, containing consensus motifs involved in replication and a CP in the C-terminal region, and ORF2 (1.0 kb) encoding a movement protein. The genomic RNA of ASPV is about 9,300 nt and contains five open reading frames (ORFs) and two untranslated (UTR) regions at 5' and 3' ends. ORF1 encoded a polyprotein for viral replication, ORF2-ORF4 encoded the triple gene block proteins, which are involved in cell-to-cell movement, and ORF5 encoded a coat protein.

Early studies showed that these viruses had differentiations in molecular, serological and biological characteristics, which were somewhat related to their geographical or host sources. Previous surveys indicated that 86.93% of pear trees in northern China were infected by ACLSV, ASGV and ASPV, and even for some varieties, the infection rate was almost 100% (Wang, 1994). In the present study, the incidence of these viruses and a viroid (*Apple scar skin viroid*, ASSVd) on a wide range of varieties collected from different regions was evaluated by RT-PCR. The three viruses were molecularly characterized based on their CP and/or MP sequences

of selected isolates. This is the first overall evaluation of the sanitary status of pear plants grown in China. These results will contribute to the further use of virus-free materials for the control of these viral diseases and the understanding of their molecular evolution.

## Materials and Methods

**Pear samples:** Between 2001-2011, field surveys were carried out to evaluate the incidence of viral diseases on pear plants grown in China. The viral disease-like symptoms were recorded. Leaf samples of 410 pear trees from 18 orchards in nine provinces were collected and tested for the presence of ACLSV, ASGV and ASPV by RT-PCR.

**RNA extraction and reverse transcription-polymerase chain reaction:** Total RNA extracted from 0.1 g leaves with the cetyltriethylammonium bromide (CTAB) method (Li *et al.* 2008) or an improved cellulose SiO<sub>2</sub> (unpublished) method was used as a template for the detection of target viruses and a viroid by RT-PCR. The primer set 5'-GGAATTTACACGACTCCTAACCTCC-3'/5'-CCCGCTGTTGGATTTGATACACCTC-3' was used for the detection of ASGV (Yoshikawa, 1992) A52 5'-CAGACCCTTATTGAAGTCGAA-3'/A53 5'-GGCAACCC-TGGAGCAGA-3' for ACLSV (German, 1990) and D-F 5'-ATGTCTGGAACCTCATGCTGCAA-3'/D-R 5'-TTGGGATCAACTTTACTAAAAAGCATAA-3' for ASPV (Menzel, 2002). Primer set M<sub>4</sub>-T 5'-GTTTTCCCAGTCACGAC(T)<sub>15</sub>-3'/ ASGV-F 5'-GGGCTTCACAGGCAATCAG-3' was used for the amplification of 3' end of ASGV genome. Primer sets 5'-GTACATGAGTAACTCGAGCC-3' / 5'-GTACATGAGTAACTCGAGCC-3' (Jelkmann and Keim Konrad, 1997) and MP-F 5'-GTG-TGTAAGCATATTAGG-3'/MP-R 5'-CTACACCCTAACCTAATG-3' were designed based on sequences available in GenBank and were used for the amplification of complete CP and MP genes of ASPV, respectively. The primer set assvdF CAGCAC-CACAGGAACCTCACGG/ assvdR CTCGTCGTCGACGAAGG was used for the detection of ASSVd. The cDNA synthesis was performed using 6-mer random primers (TaKaRa, Dalian, China) and M-MLV reverse transcriptase (Promega, Madison, USA) at 42°C for 1 h. PCR reactions were taken in a 25 µl volume with reaction mixtures containing 2.5 µl of 10×PCR buffer, 0.5 mM of each dNTP, 0.5 mM of each primer, one unit of *Taq* DNA polymerase (TaKaRa, Dalian, China), and corresponding templates. The PCR reaction conditions were as follows: 94°C for 5 min, followed by 35 cycles of denaturation for 30 s at 94°C, annealing for 1 min at 52-56°C (variable depending on primer sets used) and extension for 1 min at 72°C, and final extension for 10 min at 72°C.

**Cloning, sequencing and sequence analysis:** The target PCR products were ligated into pMD18-T vector (TaKaRa, Dalian, China). The plasmids with target fragments were verified by enzyme digestion with *EcoR* I and *Sac* I (TaKaRa, Dalian, China). At least three positive clones of each product of the studied viruses were sequenced by Genscript Corporation (Nanjing, China). The nucleotide (nt) and deduced amino acid (aa) sequences were compared using DNAMAN (Version 5.2.9, Lynnon BioSoft,

Quebec, Canada). Multiple sequence alignment was performed using the Clustal X program (<http://www.clustal.org>) (Chenna *et al.*, 2003). The phylogenetic tree was derived by importing the aligned sequences (produced with Clustal X) into the MEGA 3.1 and constructed using the neighbor-joining method with 1,000 bootstrap replicates (Kumar *et al.*, 2004). Some sequences available in GenBank were referred. The previously reported sequences of ACLSV isolates from sand pear were also briefly analyzed to show overall molecular characteristics of three viruses in pear in China.

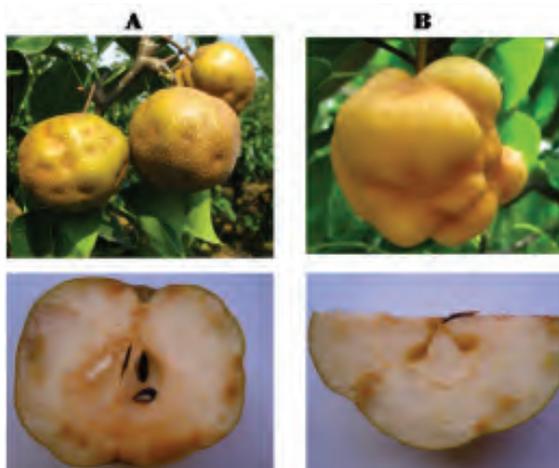


Fig. 1 - The malformation (upper panel) and stone pitting (lower panel) of fruits of *Pyrus pyrifolia* cv. Cuiguan. A. Fruits positive to ASPV and ASGV; B. Fruits positive to ASPV, ASGV and ACLSV.

## Results

**The effect of viral diseases on pear and infection status of viruses in pear grown in China:** During the growing seasons from 2001-2011, field surveys were carried out to evaluate the incidence of viral diseases on pear plants grown in China. The most frequently found viral disease-like symptoms include chlorotic leaf spot, leaf ring spot, leaf vein yellowing and mosaic, which usually occurred on young leaves. In a few orchards, some trees top-grafted for variety replacement showed uneven reddish leaves, leaf curling, and even decline and death of the whole tree. A few cultivars at some orchards, above all sandy pear grown widely in southern China, showed serious fruit malformation with stone-pitting like symptoms (Fig. 1). In order to understand the sanitary status of pear trees grown in China and the association of symptoms observed with virus infection, a total of 410 pear trees were tested for the presence of ACLSV, ASGV, ASPV and ASSVd by RT-PCR. The infection rates of ASGV, ACLSV, ASPV and ASSVd were 70%, 57.4%, 43.2% and 14.5% (28/193), respectively, and over 95% tested pear varieties were infected by one or more of these viruses. It was noticed that ACLSV was usually associated with leaf ring spot and all fruit stone-pitting, declining and vein yellowing symptoms were associated with ASPV infection, respectively. The mixed infection of two or more viruses and viroids occurred commonly in pear plants (Tab. 1).

Tab. 1 - The mixed infection status of three viruses and a viroid on different types of pear

Viruses #	Infection status of viruses and a viroid on different types of pear						
	Positive plants	Positive cultivars	<i>P. pyrifolia</i>	<i>P. communis</i>	<i>P. ussuriensis</i>	<i>P. sinkiangensis</i>	<i>P. broschneideri</i>
G+P	42/193*	29/71	21/39	2/23	6/32	2/20	11/77
G+S	50/193	27/71	8/39	0/23	15/32	3/20	24/77
G+d	20/193	16/71	3/39	1/23	5/32	2/20	9/77
P+S	22/193	14/71	11/39	0/23	3/32	3/20	5/77
P+d	8/193	7/71	2/39	1/23	1/32	2/20	2/77
S+d	10/193	9/71	1/39	2/23	1/32	3/20	3/77
G+P+S	17/193	12/71	7/39	0/23	3/32	2/20	5/77
G+P+d	6/193	6/71	1/39	0/23	1/32	2/20	2/77
G+S+d	6/193	5/71	0/39	0/23	1/32	2/20	3/77
G+P+S+d	2/193	2/71	0/39	0/23	0/32	2/20	0/77

Samples listed in the table above were only those from two collections of pear germplasm

#G:ASGV; P:ASPV; S:ACLSV; d:ASSVd.

\*number of positive plants number of test plants.

### Molecular variability of three viruses from pear in China

#### 1. The molecular variability of CP and MP genes of ASPV from pear plants

A total of 169 cDNA clones of complete CP gene derived from 31 isolates and 95 cDNA clones of complete TGB genes derived from 52 isolates were sequenced. Results showed that ASPV isolates from pear plants were highly divergent, and their CP genes shared identities of 66.4~99.8% at nt level and 68.6~99.8% at aa level, respectively. Phylogenetic analysis of globe ASPV isolates revealed the existence of at least six groups for CP sequences (Fig. 2a). The CP sequences of all Chinese ASPV isolates clustered into five groups (Fig. 2b). It was noticed that only five isolates obtained in this study clustered in the same group with apple isolates reported previously. Two previously sequenced ASPV isolates (KL1, KL9) from Korla pear (*Pyrus sinkiangensis*), which is specifically grown in Xinjiang province, north-western China, showed low identities with all the other isolates, ranging from 66.4 to 72.2% (nt) and 68.6 to 78.20% (aa), formed a separate group Gp5. Some CP genes had continuous nucleotide acid inserts or deletions, which was correlated to their phylogenetic positions. Moreover, the (CP) sequences from 13 out of 31 ASPV isolates showed complicated molecular population structures, consisting of two or four distinct variants. Clones from one isolate YN-MRS were highly divergent, sharing 72.4%-99.7% nt identity, and were divided into three groups 1, 3, and 4. The isolate was collected from a pear tree that had declined rapidly. The whole TGB gene

shared identities of 75.1-99.9% at nt level. Phylogenetic analysis of MP sequences of ASPV revealed the presence of four groups (Fig. 2c). The genetic distances inter groups were  $0.128\pm 0.030$  to  $0.175\pm 0.043$ . Two clones of isolate YN-MRS shared only 78.4% nt identity, and two clones of isolate HN-HBL shared only 77.9% nt identity.

### 2. *The molecular variability of CP gene of ASGV from pear plants*

The partial CP gene (482 nt covering 62.7% of complete CP gene) of 45 ASGV isolates collected from 43 pear varieties grown in five provinces in China was sequenced. Results showed that the CP genes of different ASGV isolates were also highly divergent, sharing 86.7-99.4% nt identities and 94.3-100% identities at aa level, and having the highest divergence of 13.3% at nt level. Phylogenetic analysis based on nt sequences of their CP genes revealed the existence of two groups, which were divided into two sub-groups, respectively. The complete CP gene of 17 ASGV isolates from pear showed 89.5-98.7% nt identities. However, the genetic distance of intra isolates was very low (less than 0.02), indicating a much less complicated population structure.

### 3. *The molecular variability of CP gene of ACLSV from pear plants*

Like the previously reported results, the sequences of CP genes of 22 ACLSV isolates from pear showed a high divergence, with 87.3-100% identities at the nucleotide (nt) level and 92.7-100% identities at the amino acid (aa) level. Phylogenetic analysis on the aa sequence of CP showed that the analyzed ACLSV isolates fell into different clusters and all isolates from sand pear were grouped into a large cluster (I) which was divided into two sub-clusters (Song *et al.*, 2011).

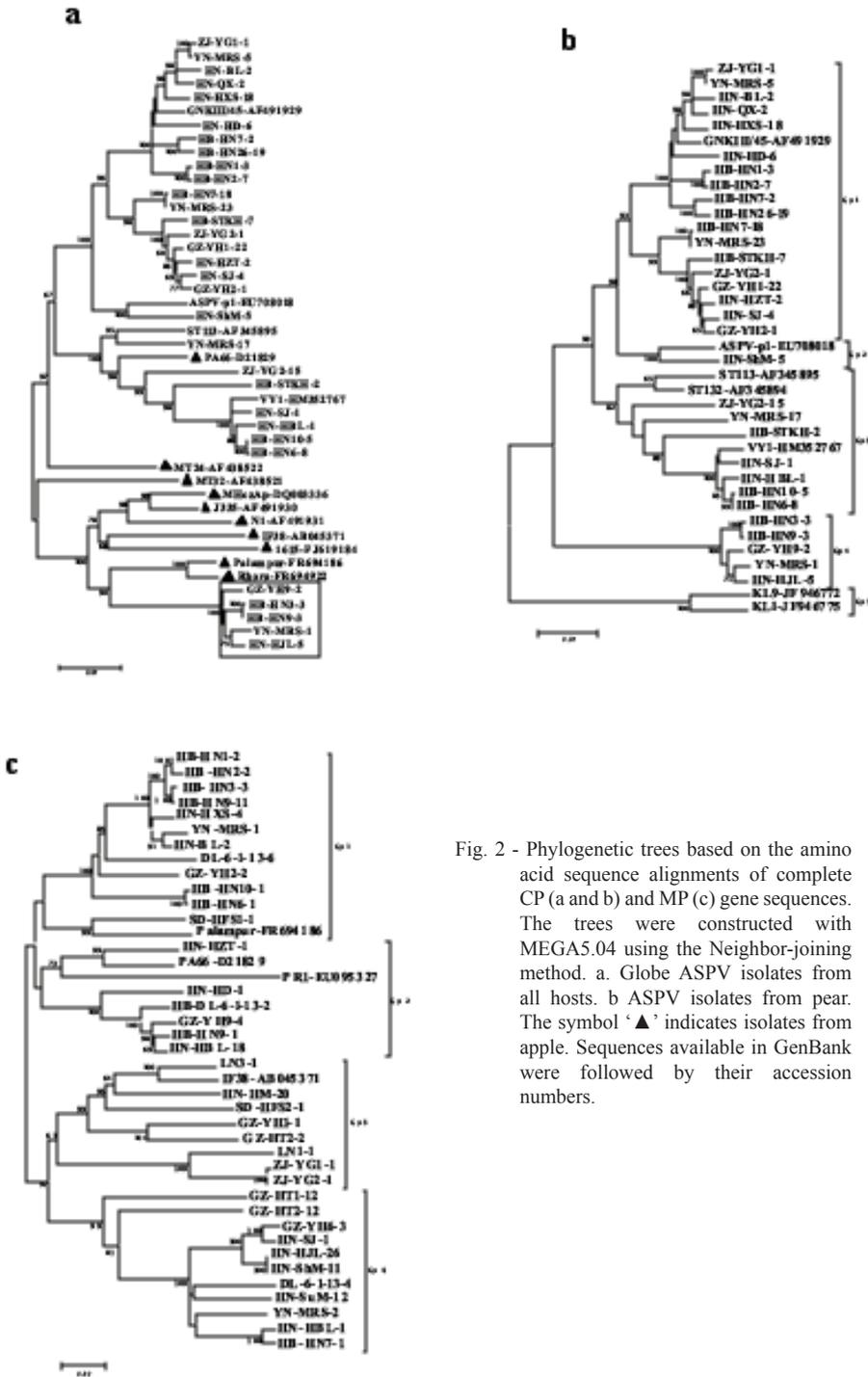


Fig. 2 - Phylogenetic trees based on the amino acid sequence alignments of complete CP (a and b) and MP (c) gene sequences. The trees were constructed with MEGA5.04 using the Neighbor-joining method. a. Globe ASPV isolates from all hosts. b ASPV isolates from pear. The symbol '▲' indicates isolates from apple. Sequences available in GenBank were followed by their accession numbers.

## Discussion

This is the first study on the incidence of viruses in wild types of pear plants grown and collected in China. Previous biological indexes showed that the infection of viruses was very common in pear plants grown in northern China (Wang, 1994). However the mixed infection status and its association with viral disease development are rarely known. The association of specific symptoms with virus infection observed in this study confirmed that ASPV and ACLSV infection could induce severe viral disease in some varieties of pear (Cembali, 2003; Jelkmann, 1996). Meanwhile, we also found that the infection of these viruses did not always develop the disease in the same pear variety, suggesting that there could be pathogenic or strain divergence among isolates of these viruses. Although, the mixed infection of three viruses in *Pyrus pyrifolia* cv. Cuiguan caused very severe fruit malformation and stone pitting symptoms, the biological characteristics of these viruses (especially ASPV) implicated in their virulence should be studied further. The results obtained in this study revealed an unusually high molecular divergence in the ASPV population in pear in China. A further study to address the biological functions of such a high divergence within ASPV isolates will contribute to understanding the virus's evolution and pathogenicity.

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## DETECTION AND IDENTIFICATION OF *PEAR BLISTER CANKER VIROID* OCCURRING IN PEAR TREES IN CANADA

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### Abstract

A survey was conducted in Canada for the presence of *Pear blister canker viroid* (PBCVd) in pear trees of different origin. A total of 118 pear samples were tested and only two (Anjou Euwer and Clapp's Favorite Starkrimson) tested clearly positive, as also confirmed by RT-PCR with specific primers and sequencing of amplified products. Infection by *Apple scar skin viroid* and/or *Apple dimple fruit viroid* in all the tested samples was ruled out the basis of TPH hybridization tests with specific probes. This is the first documented report of PBCVd in Canada.

### Introduction

Pome fruit trees are natural hosts of a number of viroid species, some of which are responsible for severe diseases (Hadidi and Barba, 2011; Koganezawa and Ito, 2011; Kyriakopoulou, 2001, 2003; Di Serio, 2011; Flores, 2011). Pathogenic effects in infected hosts have been demonstrated for infections by *Apple scar skin viroid* (ASSVd) (Hashimoto and Koganezawa, 1987; Puchta, 1990; Yang, 1992), *Apple dimple fruit viroid* (ADFVd) (Di Serio, 1996; 2001b and *Apple fruit crinkle viroid* (Ito and Yoshida, 1998), which elicit fruit disorders on certain apple cultivars, and for *Pear blister canker viroid* (PBCVd) (Hernández *et al.*, 1992a), the agent of bark alteration in the pear indicator A20 (Hernández, 1992b; Ambrós, 1995). These four viroid species are classified in the genus *Apscaviroid*, family *Pospiviroidae* (Owens, 2012).

While the spread of ASSVd and AFCVd is prevalent in East Asia with limited incidence in North America and Europe (Hadidi and Barba, 2011), ADFVd has been reported only in Italy (Di Serio, 1996, 2001), Lebanon (Choueiri, 2007) and Japan (He, 2010). In contrast, the occurrence of PBCVd, initially reported in France and Spain (Desvignes, 1970; Hernández, 1992b; Ambrós, 1995), later extended to Italy, Greece, Tunisia and Australia (Loreti, 1997; Kyriakopoulou, 2001; Malfitano, 2004; Joyce, 2004; Hassen, 2006). However, Desvignes, (1999) highlighted that the dis-

tribution of PBCVd was probably underestimated. In line with this prediction, large scale surveys conducted in the last few years using a molecular approach based on tissue printing hybridization (TPH) (Lolic, 2007), revealed the presence of PBCVd also in Malta (Attard, 2007), Bosnia and Herzegovina (Lolic, 2007) and Turkey (Yesilcollou, 2010), thus supporting a widespread presence of this viroid in the Mediterranean basin (Di Serio, 2010).

In order to investigate whether PBCVd is also present in other regions of the world, a survey was conducted in Canada for the presence of this viroid in pear trees of different origin. Here we show that at least two pear samples collected in southwestern Ontario were infected by PBCVd which was identified by TPH, RT-PCR (retro-transcriptase real time polymerase chain reaction) and sequencing.

### **Materials and Methods**

Pear leaf samples were collected from the southern Ontario production area and were tested for PBCVd infection by TPH according to Lolic *et al.* (2007). Tissue prints were carried out by pressing cut ends of petioles from three different leaves of each tested tree onto Hybond-N+ (Roche Diagnostics GmbH, Germany) membranes. At least two different trees of each cultivar were tested. TPH assays were carried out in autumn 2010 on 118 pear samples, and in spring 2011 on 25 samples selected on the basis of the results of the first assay. Total nucleic acid preparations from emerging leaves were prepared as described (Lolic, 2007) and analyzed by RT-PCR according to Malfitano *et al.* (2004). The nature of the infecting viroid was conclusively established by sequencing amplified cDNAs products from each positive tree. Phylogenetic relationships between sequenced cDNAs and previously reported variants were performed as reported previously (Yesilcollou, 2010).

### **Results and Discussion**

Initial screening was performed in autumn 2010 by Agriculture and Agri-Food Canada by tissue printing hybridization (TPH) using a digoxigenin-labeled riboprobe specific for detecting PBCVd. A total of 118 pear samples were screened and two cultivars (Anjou Euwer and Clapp's Favorite Starkrimson) tested clearly positive for PBCVd. In early spring 2011, 25 samples, including two samples that had tested positive and 23 additional samples generated a weak hybridization signal in the first analysis, were thus re-screened by TPH. The possible infection by PBCVd, or a closely related viroid for the cultivars Anjou Euwer and Clapp's Favorite Starkrimson was confirmed. In contrast no hybridization signal was obtained from the other tested samples, including the healthy ones. The confirmation of PBCVd infection was obtained at the Istituto di Virologia Vegetale (Italy) and the Canadian Food Inspection Agency by testing independently total nucleic acid (TNA) preparations from both TPH-positive samples by RT-PCR using PBCVd specific primers, and by direct sequencing of the amplification products. Amplified cDNAs of expected sizes obtained from the cultivars Anjou Euwer and Clapp's Favorite Starkrimson (Fig. 1), and the sequence obtained shared a

94-97% sequence identity with the PBCVd reference variant (NC\_001830), conclusively showing that they had been infected by this viroid. In contrast, TNAs from additional five samples, selected among those testing negative to the TPH analyses, did not generate any amplification products when assayed by RT-PCR, confirming the absence of PBCVd in these samples (Fig. 1). In parallel experiments, possible infections by ASSVd and ADFVd in the 118 tested samples were ruled out on the basis of TPH hybridization with specific probes performed as previously reported (Lolic, 2007).

In a previous study (Yesilcollou, 2010), it was shown that PBCVd variants

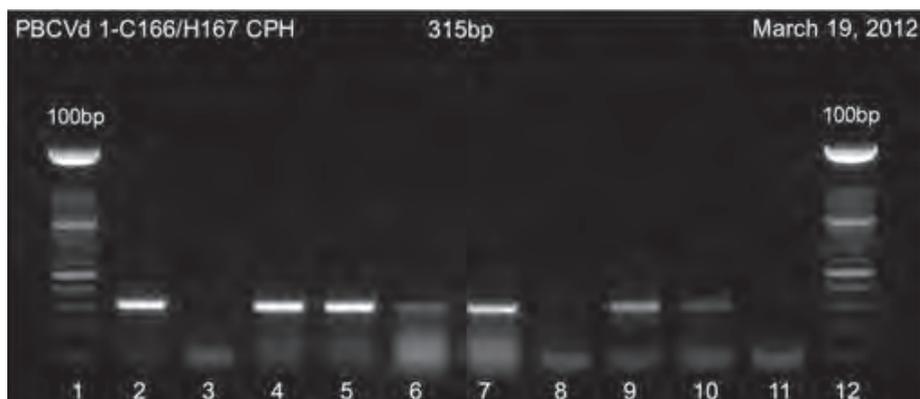


Fig. 1 - Agarose gel electrophoresis analysis of DNA products amplified from infected pear leaf samples by RT-PCR. Lanes 1 and 12, DNA molecular marker (Roche XIV); lanes 2, 9, 10: PBCVd positive control; lanes 3 and 8, negative control (healthy pear leaves); lanes 4 and 5: cultivar Clapp's Favorite Starkrimson; lanes 6 and 7: cultivar Anjou Euwer; lane 11: no template control.

in databases clustered in two main groups of a phylogenetic tree, according to their geographic origin and the host species they came from. Most variants from Greece fell into a single phylogenetic group containing all variants from apple, wild apple and wild pear species, while all variants from Turkey, Australia and Europe, including variants from pear and nashi and one from quince, clustered in a separate group. Interestingly, when a similar phylogenetic tree is generated including the two new PBCVd variants from Canada, the same two phylogenetic clusters are generated, with these new variants clustering together with those reported previously from pears cultivated in Europe, Turkey and Australia (data not shown).

This is the first report of PBCVd in Canada and represents an urgent call for the prompt extension of similar analyses to other pear cultivations in the country.

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## A REVIEW OF VIRUSES INFECTING *ACTINIDIA*

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Kiwifruit (*Actinidia deliciosa*) was introduced in New Zealand in 1904 from seeds imported from China. Since then seeds and scionwood have been imported to New Zealand from China, to increase the gene pool for breeding purposes. The first virus naturally infecting kiwifruit, *Apple stem grooving virus*, was identified following symptoms observed in imported plants growing in quarantine (2003). Three novel viruses, two vitiviruses, and a citrivirus closely related to *Citrus leaf blotch virus*, have since been identified from plants originating from the same importation. All belong to the family *Betaflexiviridae*. Members of the family *Bromoviridae*, *Alfalfa mosaic virus* and *Cucumber mosaic virus* (CMV), have been detected in the field. In New Zealand, these were mostly limited to *Actinidia guilinensis* and *A. glaucophylla* seedlings, while CMV was detected in *A. deliciosa* in Italy. These viruses are cosmopolitan, and weeds provide a reservoir for infection. From the same family, *Pelargonium zonate spot virus* (PZSV) has been detected in Italy associated with severe symptoms on leaves and fruit. Four viruses appear to have a limited effect on kiwifruit. Two tobamoviruses, *Ribgrass mosaic virus* and *Turnip vein clearing virus*, are probably present worldwide in kiwifruit and are also present in *Plantago* spp., a common weed in kiwifruit orchards. The *Cucumber necrosis virus* family *Tombusviridae* has been detected in kiwifruit at very low titre without apparent symptoms. Additionally, a novel *Potexvirus* has been transmitted to herbaceous indicators from three kiwifruit plants, but direct detection from the original plants was unsuccessful. Finally, *Cherry leaf roll virus* (CLRV), family *Secoviridae*, has been detected in New Zealand on kiwifruit and also in *Rumex* spp. growing below the infected vines. Major symptoms were observed on the kiwifruit, including leaf spots, fruit malformation, reduced yield, bark cracking and cane wilting. The symptoms are similar to those observed in Italy from PZSV infection.

## **PELARGONIUM ZONATE SPOT VIRUS INFECTING KIWIFRUIT PLANTS IN ITALY**

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*Pelargonium zonate spot virus* (PZSV) is the single member of the *Anulavirus* genus (Fam. *Bromoviridae*) first isolated from tomato in Italy and later reported also in Spain, France, USA and Israel. Up to now PZSV has been known to naturally infect only herbaceous hosts such as tomato, pepper, artichoke and common weeds often symptomless. Symptoms on leaves and fruits of infected tomato plants are characterized by line patterns, chlorotic and necrotic rings, together with plant stunting, leaf malformation and poor fruit set, which often result in plant death as infected cells show severe cytopathological alterations. The virus is transmitted by mechanical inoculation, grafting, and through seeds by means of infected pollen carried on the bodies of thrips. In May 2011 plants of kiwifruit (*Actinidia chinensis*) cv. Hort16A, exhibiting viral symptoms, were observed in two orchards in the regions of Faenza, and Emilia-Romagna. Symptoms included chlorotic and necrotic rings on leaves and depressed areas on the fruits with a consequent deformation of the berries. The causal agent was successfully transmitted to indicator plants, such as *Chenopodium quinoa*, *Nicotiana benthamiana*, *N. glutinosa* and *N. tabacum*, by mechanical inoculation. Viral particles with a diameter of about 30 nm were observed on viral purifications obtained from *C. quinoa* infected leaves. Random amplification and sequencing of nucleic acids isolated from the purified virus, led to the identification of a short segment showing high nucleotide identity with 5' end of PZSV RNA2. RT-PCR analyses, performed using a PZSV primer pair, specifically identified the virus in all the indicator hosts and in leaves and fruits collected from all symptomatic kiwifruit plants. The new PZSV isolate was characterized by sequencing and by ultrastructural and immunotransmission electron microscopy investigations. Moreover, in order to determine the effect of the virus on cellular water compartmentation, tissue metabolic activity and overall quality, fruits and leaves from infected and healthy plants were compared by NMR measurements (Proton transverse relaxation time), isothermal calo-respirometry (metabolic heat production) and visual appearance using a computer vision system.

## **ASSOCIATION OF PHYTOPLASMAS AND ROD SHAPED BACTERIA WITH A CITRUS DISEASE OF UNKNOWN ETIOLOGY IN THE STATE OF BAJA CALIFORNIA SUR, MEXICO**

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Multiple symptoms of yellow-type diseases were observed in citrus trees from various citrus growing areas in the state of BCS. Leaf malformations, chlorosis, blotching and necrotic spots, yellowing of leaf veins and shoots, necrotic leafstalks and shoots, leaf dropping and dieback, were reported for distinct citrus diseases worldwide and related with phytoplasmas and other vascular pathogens. To determine the possible causal agent/agents of these citrus diseases, samples from symptomatic lemon, sweet orange, mandarin and grapefruit trees were collected and processed for scanning electron microscope (SEM) analysis. Specimens from leaf midribs, leafstalks, young leaflets and stems from symptomatic plants were prepared and observed in a Hitachi S-300N SEM. Both phytoplasmas and rod-shaped bacteria were observed in phloem tissue of symptomatic plants. The most abundant phytoplasma was detected in the samples of mandarin (south of BCS) where bacteria were not detected. On the other hand, in samples of lemon and orange trees collected from northern BCS along with phytoplasmas, some rod-shaped bacteria were observed with a specific smooth outer surface. In some sieve tubes their concentration was very high. Phytoplasma size ranged from 500 to 1,000 nm, and bacterial size reached 3,000 x 500 nm, similar to former prokaryote sizes reported in the case of citrus greening. A very high level of phytoplasma (but not of rod shaped bacteria) was also registered in acacia species, as well as in some herbaceous plants growing in the same plots, which suggests that these species could be wild hosts of this pathogen. In some lemon and orange samples phytoplasma detection was also achieved by a nested PCR technique using phytoplasma specific primers. The work is in progress to confirm all the cases of phytoplasma infection detected by SEM, to identify both pathogens and prove that the mixed infection of the two prokaryotes could be the basis of the disease etiology.

## DETECTION OF VIRUSES INFECTING APPLE IN NORTH WESTERN HIMALAYAN REGION

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Apple is cultivated on a commercial scale in the hill state of Himachal Pradesh situated in the North Western Himalayan region of India. The economy of Himachal Pradesh is largely dependent on apple cultivation. Of late, viruses have been observed to be a major constraint in the declining apple productivity in the state. Infection of *Apple chlorotic leaf spot* (ACLSV), *Apple stem pitting virus* (ASPV), *Apple stem grooving virus* (ASGV) and *Apple mosaic virus* (ApMV) have been invariably associated with commercial apple plantations of Himachal Pradesh with an incidence as high as 95 percent. Chlorotic spots turning into necrotic lesions on apple leaves were found to be the predominant symptoms. An orchard with a very high incidence of viral diseases (95 percent) was selected for conducting biological and serological detection of apple viruses. Serological detection through DAC and DAS-ELISA resulted in the identification of ACLSV, ASPV, ApMV and ASGV in the form of mixed infections in all the samples of 10 cultivars randomly marked for assays. Biological detection of one of the isolates containing a mixed infection of ASGV, ACLSV, ASPV and ApMV on herbaceous hosts resulted in the production of symptoms on *Chenopodium quinoa*, *Phaseolus vulgaris*, *Nicotiana glutinosa* and a few other hosts. Detection on woody indicators (Virginia crab, *M. platycarpa*, Spy 227, Jay Darling and Russian Clone) under field conditions through double grafting, grafting cum budding and double budding of inoculators and indicator budwood resulted in the production of typical viral symptoms on leaves in *M. platycarpa*, Spy 227, Jay Darling and Russian Clone. Swelling and necrotic symptoms were produced at the graft union in Virginia crab and Spy 227 indicators, respectively. Periodic detection of these viruses revealed that leaf samples collected during the months of March to May were found to be suitable for the ELISA of ASGV, ACLSV and ASPV whereas petals were the best source for ApMV detection. Association of ASGV infection in an apple tree was also confirmed by RT-PCR assay. Phylogenetic analysis of the coat protein gene of the Shimla ASGV isolate revealed its 100 per cent sequence similarity with Serbian isolate. Serological indexing for virus free clones resulted in the selection of 20 trees from 11 cultivars free from infection of ASGV, ACLSV, ASPV, ApMV infection and *Tomato ringspot virus* (ToRSV) in an ELISA test of 60 symptomless trees from 20 cultivars. These virus tested trees were selected to serve as important mother trees for use in raising healthy nursery plants.

## **PHYTOPLASMA DETECTION AND IDENTIFICATION IN KIWI PLANTS AND POSSIBLE CORRELATION WITH *PSEUDOMONAS SYRINGAE* PV *ACTINIDIAE* PRESENCE**

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### **Abstract**

Surveys were carried out during 2011 and 2012 in kiwi plantations located in North and Central Italy to verify the presence of phytoplasmas in *Pseudomonas syringae* pv *actinidiae* infected kiwi orchards. Samples from 72 kiwi trees were collected in 2011 and 2012 from *Actinidida chinensis* and *A. deliciosa*. Phytoplasma infected plants showed in some of the branches a premature reddening of leaves downward curled and crinkling; the green variety show also leaf reddening. These samples showed negative results for Psa presence while phytoplasmas belonging to groups 16SrI, 16SrXII and 16SrX were identified in single or in mixed infection after RFLP analyses of generic or specific amplicons from 16S rDNA. The phytoplasmas were present in diverse combinations in all the tested orchards in 68% of tested plants. The group more often identified was aster yellows (16SrI) (26%) followed by the mixed infection of aster yellows and apple proliferation (16SrX) groups (11%) or by apple proliferation (10%). Further work is in progress to verify economic impact of phytoplasma diseases in kiwifruits.

### **Introduction**

The quick, extensive and rapid spreading of the *Pseudomonas syringae* pv *actinidiae* (Psa) disease in all kiwi growing areas worldwide (Takikawa *et al.*, 1989; Serizawa *et al.*, 1989; Koh *et al.*, 1994; Balestra *et al.*, 2008; 2009; 2011; Ferrante and Scortichini, 2009; Argibay *et al.*, 2011; Everett *et al.*, 2011; Bastas and Karakaya, 2012) led to the verification of the possible involvement of phytoplasmas beside the dissemination of the new Psa strain (Vanneste *et al.*, 2010; 2011a; 2011b; Ferrante *et al.*, 2010; Scortichini *et al.*, 2012) clearly involved in the epidemic, considering that phytoplasmas could also a modify susceptibility of kiwi plants to the disease. Surveys were therefore carried out during 2011 and 2012 in kiwi plantations located in northern and central Italy to verify the presence of phytoplasmas in Psa infected kiwi orchards.

## Materials and Methods

Preliminary tests were carried out on 12 samples collected in August 2011 from samples collected in the spring and infected by Psa (A. Calzolari, personal communication). Samples from 72 kiwi trees were also collected in October 2011 and April-June 2012 in three of the main kiwi-growing regions of Italy (Emilia-Romagna, Lazio and Veneto). Both *Actinidia chinensis* and *A. deliciosa* (gold and yellow varieties, respectively) were sampled in Psa infected localities from plantations in Emilia-Romagna and from representative orchards in the other two regions. Symptoms in samples collected in the spring were often represented by drying of the shoots and poor growth (Fig.1A) due to the presence of Psa infection. The most extensive survey was carried out in Emilia Romagna where 50 plants were sampled at



Fig. 1 - Branches (on the right) and shoot of kiwi showing symptoms of reddening and rolling on the leaves and drying of the shoots with reduced growth respectively. The plant of the right is also infected by Psa.

the end of October 2011, 36 of *A. deliciosa* and 14 of *A. chinensis* showing symptoms of yellowing or reddening on the leaves that are downward (Fig.1B).

Psa isolation was carried out in NSA medium followed by HR tests in tobacco and by PCR with specific primers (Koh and Nou, 2002). Phytoplasma detection was carried out by nested-PCR assays on phloematic tissues. Leaf and young branch samples were collected and midribs and phloem scrapes were employed for nucleic acid extraction following reported procedures (Prince *et al.*, 1993). Polymerase chain reaction assays were then carried out on 20 ng of template DNA with primers P1/P7 (Deng and Hiruki, 1991; Schneider *et al.*, 1995) followed by primers R16F2n/R2 (Gundersen and Lee, 1996) in nested PCR assays using amplicons diluted 1:30 with sterile distilled water as template. Further nested-PCR assays were performed

using the general 16R758f/16R1232r (=M1/M2) (Gibb *et al.*, 1995) and group specific primers R16(I)F1/R1 (Lee *et al.*, 1994) and R16(X)F1/R1 (Lee *et al.*, 1995). A tube with reaction mixture devoid of DNA template was included as a negative control in each PCR reaction. Reference phytoplasma strains employed in periwinkle were: RuS, rubus stunt (ribosomal subgroup 16SrV-E); LNS1, European stone fruit yellows (ribosomal subgroup 16SrX-B); STOL, stolbur (ribosomal subgroup 16SrXII-A); AY, aster yellows (ribosomal subgroup 16SrI-B). All PCR amplifications were performed following published parameters (Schaff *et al.*, 1992). PCR products were electrophoresed in 1.0% agarose gel, the gel was stained with ethidium bromide and visualised under UV light. RFLP analyses with *TruI*, *RsaI*, and *SspI* were carried out on R16(I)F1/R1 and R16(X)F1/R1 amplicons. Digestion was carried out for 16-30 hrs at 37 or 65°C according with the manufacturer instructions for each enzyme. The products were separated by electrophoresis in 7% polyacrylamide gel, and visualised under an UV transilluminator.

## Results and Discussion

Only five out of the twelve Psa infected plants resulted to be negative to the analyses to verify phytoplasma presence and stolbur phytoplasmas were mainly identified in single or mixed infection. The 50 samples collected in October from Jin Tao and Hayward varieties confirmed the phytoplasma presence in high percentages. Phytoplasma-positive plants showed a premature reddening leaves; the green variety (Hayward) also showed leaf reddening (Fig. 1B) while in Jin Tao there was a general plant decline and yellowing of the leaves. Scattered plants also showed other symptoms such as an irregular shape of the non lignified branches and reddening shoot proliferation from the rootstock. These samples showed negative results for the presence of Psa while the presence of phytoplasma was confirmed groups 16SrI, 16SrXII and 16SrX were identified in single or in mixed infection after RFLP analyses of generic or specific amplicons (Fig. 2).

The various phytoplasmas were present in different combinations in all the tested orchards in the majority of the tested plants (68%) (Fig. 3); phytoplasma group that was most often identified was the one of aster yellows (16SrI) (26%) followed by the mixed infection of aster yellows and apple proliferation (16SrX) groups (11%) or apple proliferation group (10%).

Considering the three Italian regions surveyed, it appears that the majority of negative results were obtained in Emilia-Romagna (Fig. 4), however the large majority of these negative results were obtained in a single orchard of the Hayward variety.

The presence of stolbur phytoplasmas in kiwi has been reported in Italy in plants showing small and rolled leaves with vein necrosis (Marzachi *et al.*, 1999), further reports have indicated the presence of several phytoplasma groups (Bertaccini *et al.*, 2011). The results of the present survey confirm the previous reports; work is in progress to evaluate if the presence of phytoplasma may interfere with the kiwi's susceptibility to canker disease.

Surveys were carried out in kiwi plantations located in northern and central

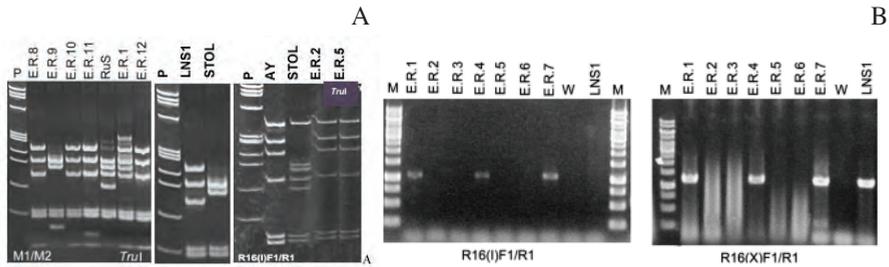


Fig. 2 - A shows results of RFLP analyses on amplicons obtained from kiwi samples (E.R.) after amplification with primers listed at the bottom of each figure with TruI. Reference strains: RuS, rubus stunt phytoplasma (16SrV-E); LNS1, European stone fruit yellows (16SrX-B); STOL, stolbur (16SrXII-A); AY, aster yellows (16SrI-B). P, marker ΦX174 HaeIII digested, fragment sizes (from top to bottom): 1,353; 1,078; 872; 603; 310; 281; 271; 234; 194; 118, and 72. B shows results of nested PCR amplification of kiwi samples with specific primers for groups 16SrI and 16SrX respectively (left to right). M, marker 1 kb DNA ladder, W, sterile distilled water. Other abbreviations are as in A.

Italy to verify the presence of phytoplasmas in Psa infected orchards. Analyses carried out on samples collected in May from plants showing severe symptoms of twig dieback and canker Psa and phytoplasmas were identified. A total of 72 samples were collected mainly from plants of the Jintao and Hayward ultivars. On some of the branches plants showed

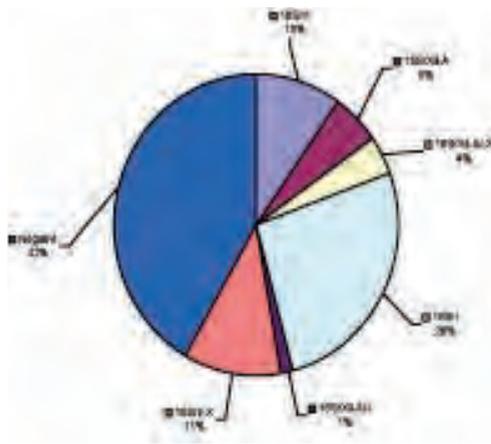


Fig. 3 - Collective results of phytoplasma identification in samples from 72 kiwi plants collected in Italian orchards infected by Psa.

premature reddening of downward curled and crinkling leaves. The green variety (Hayward) also showed leaf reddening while in cv. Jintao (yellow) there was a general plant decline and yellowing of the leaves. Phytoplasma presence was confirmed in 68% of the tested plants mainly in the cv. Jintao and groups 16SrI, 16SrXII and 16SrX were identified in single or in mixed infection. Work is in progress to evaluate the influence of phytoplasma in the epidemic of canker disease.

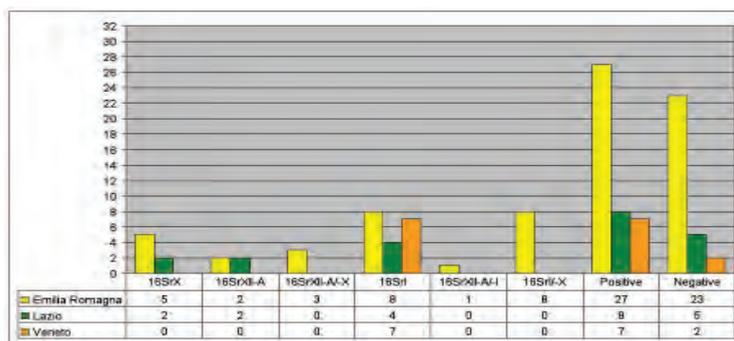


Fig. 4 - Phytoplasma distribution in the kiwi samples collected in the three regions surveyed: 16SrX=apple proliferation group; 16SrXII-A=stolbur group; 16SrI=aster yellows group.

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## **INCIDENCE OF *FIG LEAF MOTTLE-ASSOCIATED VIRUS* AND *FIG MOSAIC VIRUS* IN EASTERN PROVINCE OF SAUDI ARABIA**

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Fig plant, *Ficus carica* L., is grown in Saudi Arabia and is being affected by fig plant mosaic diseases (*Fig leaf mottle-associated virus*, *Fig mosaic virus*). The main symptoms are chlorotic mottling, blotching and various types of leaf deformation. Samples were collected, taking into consideration the economic importance and distribution of the cultivars, from different areas of Hofuf Saudi Arabia. Each sample consisted of 10-15 leaves. Samples were labelled and stored in plastic bags at 4°C; then transferred to the laboratory, for total nucleic acid (TNAs) extraction. One hundred mg of leaf veins and or cortical scrapings were used for extraction. Samples were macerated in 1 ml of grinding buffer. TNAs were recovered with a silica-capture procedure and stored at -20°C till used. 8-10µl of TNA extracts were mixed with 1µl random hexamer primer, (Boehringer Mannheim, GbmH) (0.5 µg/µl). RT-PCR assay of leaf extracts of infected fig accession using specific primers gave positive results and non with FLMaV-2. Mixed infection of FLMaV-1 and FMV were found. To our knowledge this is the first record and identification of FLMaV-1 and FMV in Saudi Arabia. Further studies are needed to investigate fig mosaic disease throughout the country.

## THE INCIDENCE OF *PRUNUS NECROTIC RINGSPOT VIRUS* IN APRICOT AND PEACH IN SAUDI ARABIA

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Cultivated areas with fruit trees in Saudi Arabia are estimated at 233,513 h and produce more than 1.6 million tons of fruits annually. *Prunus necrotic ringspot virus* (PNRSV), *Prune dwarf virus* (PDV) and *Plum pox virus* (PPV) are the most important and common viruses infecting stone fruit trees and are present in the neighbouring countries of Jordan, Lebanon, Syria and Egypt. In spring 2009, field surveys were carried out in an area of stone fruit production (Al Juof - North of Saudi Arabia) to assess the viruses of stone fruit trees. Apricots and peaches were observed showing chlorotic rings, necrotic spots, and a shothole appearance. A total of 166 leaf samples (65 apricots and 101 peach) were collected to be analyzed for the causal virus. Double antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA) commercially available for PNRSV was performed. Results showed that 30 (11 apricot and 19 peach) out of 166 leaf samples were infected with PNRSV. Total RNA, from same samples used in ELISA test, was extracted in accordance with instructions. RT-PCR was performed using specific primers for PDV, PNRSV and PPV specific primers. RT-PCR resulted in the amplification of a 346 bp fragment as expected and was negative result for PPV and PDV. The results indicate the presence of PNRSV in apricot and peach in Saudi Arabia. The amplified fragment was sequenced and deposited in GenBank (Accession No. HM584814). The sequence was compared with PNRSV isolates and had 100% identity with AF170170 from the Czech Republic while and 97% identity for PNRSV from the USA (AF013287). Further investigations needed for other commercial orchards and nurseries. This result demonstrates the first detection of PNRSV in Saudi Arabia.

## EVIDENCE FOR 'CA. P. PYRI' AND 'CA. P. PRUNORUM' INTER-SPECIES RECOMBINATION

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The genetic diversity of three temperate fruit tree phytoplasmas; '*Candidatus* Phytoplasma prunorum', '*Ca. P. mali*' and '*Ca. P. pyri*' was established by multilocus sequence analysis. Genotyping of Spanish and Azerbaijani '*Ca. P. pyri*' isolates revealed that they possessed both '*Ca. P. pyri*' and '*Ca. P. prunorum*' genetic markers, supporting for the first time the existence of an inter-species recombination between these two species. Particularly, isolate Spa3 had *aceF* and *imp* genes of '*Ca. P. prunorum*' while its *pnp* and *secY* genes corresponded to '*Ca. P. pyri*' genotypes. Isolate Spa2 also had two genes corresponding to '*Ca. P. prunorum*' genotypes and two genes clustering within the '*Ca. P. pyri*' phylogenetic branch, whereas AZ-POI45 had only a '*Ca. P. prunorum*' *pnp* gene and three other gene markers corresponding to '*Ca. P. pyri*'. Taken together, these results indicated that these isolates of '*Ca. P. pyri*' are inter-species recombinants. Surprisingly, for the Spa2 isolate, two different *imp* sequences were detected, one corresponding to '*Ca. P. prunorum*' and the other clustering with '*Ca. P. pyri*' *imp* sequences. This could not be associated with a mixed infection as only '*Ca. P. pyri*' 16S rDNA sequence was detected. A simple recombination event between '*Ca. P. pyri*' and '*Ca. P. prunorum*' linear chromosomes can lead to the exchange of many gene markers at the same time. However, for a recombination between two phytoplasma species, they have to share a common host. Peach has been described as a common host for these two phytoplasmas. Recombination might also have occurred in a common insect vector, such as *C. pyri*, which is more polyphagous in autumn and might acquire both phytoplasmas from their respective host plant. Because none of the three '*Ca. P. pyri*' recombinants had the same MLST genotype, it is likely that the recombination event is quite frequent. Indeed, it represented three cases from two distinct geographical areas over 19 different '*Ca. P. pyri*' isolates tested. It is certainly possible that if recombination occurs between species it could also occur within species. We cannot confirm such a phenomenon because, despite incongruence between the phylogenetic trees of the different markers, the bootstrap validity levels were not high enough to consider it evident.

## EPIDEMIOLOGICAL STUDY OF APPLE PROLIFERATION IN SPAIN

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### Abstract

Apple proliferation (AP) disease caused by ‘*Candidatus Phytoplasma mali*’ is responsible for considerable economic losses in the apple orchards of Europe. In Europe two psyllid species have been cited as vectors of the phytoplasma: *Cacopsylla picta* (syn. *C. costalis*) and *Cacopsylla melanoneura*. In this study the incidence of ‘*Ca. P. mali*’ in apple orchards of three geographical regions of Spain is evaluated. The population evolution and percentage of individual cases of *C. melanoneura* and *C. picta* carriers of the ‘*Ca. P. mali*’ were determined in affected plots. Results confirmed the presence of ‘*Ca. P. mali*’ in the samples from symptomatic trees from the Basque Country and Asturias. The incidence of the disease in the affected plots ranged from 20 to 70%. The most affected plots were those planted with local cider cultivars. The surveys conducted in 2010 and 2011 showed that both vector species of *Cacopsylla* cited as transmitters of the disease were present in the Basque Country and Asturias (northern Spain). The population evolution of these species showed two peaks, one for adult re-immigrants, which in both years occurred in early April and another for new generations between June and July. The high percentage of individual carriers of the phytoplasma was obtained between the months of June and July (25-33% of positives) for *C. picta*.

### Introduction

Apple proliferation (AP) phytoplasma, classified as ‘*Candidatus Phytoplasma mali*’ (Seemüller and Schneider, 2004) causes severe epidemics and considerable economical losses in apple orchards. This phytoplasma was first identified in Italy in the 1950s (Refatti and Ciferri, 1954) is currently present in many countries of Europe, such as Germany, France, Switzerland, Poland and the Czech Republic. In the few last years an important increase in the disease has been observed in Europe. Some of the characteristic symptoms of AP are witches broom, elongated stipules, chlorosis and early leaf reddening. The fruits have a smaller size and poor quality. In Spain AP was identified in apple varieties for the production of cider in 2010.

In the last few years there has been a significant increase of the disease in Europe. This reactivation may be due to the emergence of new isolates or to the presence of more effective vectors (Jaraush *et al.*, 2000). For this reason, the identification

and control of vectors and a good understanding of the population dynamics, insect infectivity and the host plants for both phytoplasma and vectors in a specific region are of great importance.

In Europe two psyllid species have been cited as vectors of the phytoplasma: *Cacopsylla picta* (syn. *C.costalis*) and *Cacopsylla melanoneura* (Carraro *et al.*, 2001). However, their distribution, infectivity and transmission capacity vary among different geographical areas. Data from France and Switzerland indicate that though the population of *C. melanoneura* is higher than *C. picta*, the latter have a greater percentage of infective individuals and a capacity of transmission than *C. melanoneura* (Jarausch *et al.*, 2007). Studies in Italy indicate that *C. melanoneura* is the main vector of the disease (Tedeschi *et al.*, 2007). Both species remain a few months in the crop, completing their biological cycle of a generation per year in shelter plants of the forest. *C. melanoneura* is known as the psylla of the hawthorn (*Crataegus* sp) (Tedeschi *et al.*, 2002), since it is common in species of this kind, located principally in the low zones of the valleys, whereas *C. picta* is found more frequently in the mountainous zones in species of the genus *Malus* (Pedrazzoli *et al.*, 2007; Tedeschi *et al.*, 2003). Also the cicadelidae, *Fieberiella fiorii* has been mentioned as a vector of the disease, though it seems that its importance in the dissemination of the disease is minor (Tedeschi and Alma, 2006).

The aim of the present study was to determine the incidence of 'Ca. *P. mali*' in host plants and to follow the population evolution and percentage of individuals of *C. melanoneura* and *C. picta* carriers of the AP phytoplasma in the affected plots.

## Materials and Methods

**Phytoplasma detection in plants:** Apple orchards of the Basque Country, Asturias and Catalonia were surveyed to determine the presence of apple proliferation disease. Samples from symptomatic trees were analyzed by nested PCR using universal primers P1/P7 in the first step and the fO1/rO1 primers specific to the AP group in the second step (Deng and Hiruki 1991, Smart *et al.*, 1996; Lorenz *et al.*, 1995).

**Insect capture and phytoplasma detection:** During 2010 and 2011 the search for psyllid vectors of the phytoplasma *Candidatus* Phytoplasma mali, and other potential vectors was extended to different apple growing areas of Spain (Asturias, Basque Country and Catalonia). The insects were captured on yellow sticky traps (10 x 16 cm) placed in apple orchards and were replaced every 15 days. The insects were captured from February until the beginning of August. All specimens captured were separated and classified for DNA extraction and PCR analysis. DNA from insects was extracted by grinding 1-5 insects, depending on the species, following the methods used in previous studies (Garcia-Chapa *et al.*, 2005).

## Results

**Disease incidence:** PCR results confirmed the presence of ‘*Ca. P. mali*’ in the samples from symptomatic trees from the Basque Country and Asturias. The most affected plots were those planted with the local cider cultivars such as Goicoetxea and Mozoloa (Tab. 1). The phytoplasma was also identified in the cultivars Elstar, Belle de Boskoop, Reineta Gris and Verde agria (Tab. 1). The incidence of the disease in the affected plots ranged between 20 to 70%. In Catalonia some positive trees were identified immediately, but to date dissemination of the disease has not been observed up to the moment (Tab. 1). However, in a sanitary selection procedure of apple plant material, the PCR analyses showed the presence of the phytoplasma in some varieties as Fuji, Granny Smith and Early Red One (Tab. 1).

Tab. 1- Incidence of the phytoplasma ‘*Ca.P.mali*’ in commercial and experimental plots

C. PICTA	29-MAR	13-APR	27-APR	11-MAY	25-MAY	8-JUN	21-JUN	5-JUL
2010		18(2+/14)	8	11 (0+/7)	2 (0+/2)	3 (1+/1)	15	6 (2+/6)
2011			8(1+/8)	7(0+/3)		17(2+/10)	18(1+/16)	5(1+/5)

**Vector identification:** Several species of psyllids and leafhoppers were caught in the sampled plots. The two species of *Cacopsylla* cited as transmitters of the disease were identified in the Basque Country and Asturias. In Orberlaún (Basque Country), with a higher incidence of the disease, *Cacopsylla picta* and individuals of *C. melanoneura* were identified. In Siero (Asturias) the two species of *Cacopsylla* were also identified but with a lower population. In Catalonia where the disease is not present, these species have not been identified. The population evolution of these species showed two population peaks, one for adult re-immigrants, which for both years occurred in early April and another for new generations between June and July (Figure 1). The higher population of these species, know as vectors of the ‘*Ca. P. mali*’, was found in the most affected area (Orberlaún, Guipúzcoa) (Fig. 1, Tab. 2). In *C. picta*, the percentage of carriers of the phytoplasma in re-immigrant individuals was around 15%. The high percentage of positives was obtained in the new generations between the months of June and July (25-33% of positives) in the Basque Country (Tab. 2). For *C. melanoneura* no positive individuals were determined from re-immigrant population whereas in the new generation the positive individuals ranged between 25 and 100% (Data non shown). Other species of cicadellidae known as potential vectors of phytoplasmas were captured in the sampled plots. In the plots of Catalonia located very close to plum, peach and pear plots, other *Cacopsylla* species such as *C. pruni* and *C. pyri* were captured.

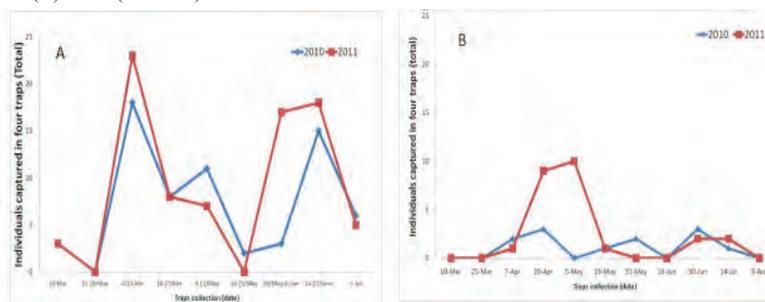
Tab. 2 - Detection of 'Ca. P. mali' phytoplasma in individuals of *C. picta* (number of positives over total analyzed) in Orberlaín (Basque Country).

<b>Incidence of <i>Ca. P. mali</i> in affected plots of apple</b>			
Locality	Variety	Incidence <sup>a</sup>	Vectors
Catalonia			
Girona	Pink Lady Golden	0,20%	No
Lleida	Golden, Fuki, Pink Lady	0,10%	No
Barcellona	Golden And Others	0,00%	No
Basque Country			
Guipúzcoa	Cider And Other Varieties <sup>b</sup>	20-70%	Yes
Asturias	Blanquina, Cooradaona, Verdialona, Regona, Raxao, Perico, Limón Montés	15-40%	Yes

**Analysis of plant materia from a selection process**

<sup>b</sup>affected Varieties: Aranzate, Errezila, Gazigorri, Gezamina, Gezamina Beltza, Goikoetxea, Areso, Manttoni, Mikatza, Txalaka, Urbin, Urbin Txiki, Urtebi Haundi, Urtebi Txiki And Verde Agria

Fig. 1- Population evolution of *C. picta* in (A) Orberlaín (Basque Country) and of *C. melanoneura* in (B) Siero (Asturias) in 2010 and 2011.



**Incidence of *Ca. P. mali* in affected plots of apple**

Locality	Variety	Incidence <sup>a</sup>	Vectors
Catalonia			
Different origin	Early red one, Fuji, Granny Smith, BP-99		

<sup>a</sup>In Asturias and Basque Country the incidence corresponds only to affected plots

<sup>b</sup>affected Varieties: Aranzate, Errezila, Gazigorri, Gezamina, Gezamina Beltza, Goikoetxea, Areso, Manttoni, Mikatza, Txalaka, Urbin, Urbin Txiki, Urtebi Haundi, Urtebi Txiki And Verde Agria

**Discussion**

Recently in Catalonia some positive trees have been identified sporadically in the plots, but dissemination of the disease has not been observed to date, probably due to the vectors species not being present. The fact that phytoplasma has also been identified in the plant material of apple trees in the process of sanitary selection, warns of a possible dissemination of the disease, the presence of the vectors spreading to other zones or other species of insects appearing with the ability to transmit the phytoplasma.

Both in the Basque Country and in Asturias, the prevalent transmitter species was *C. picta*, which is also the main vector in other countries such as Germany and Italy.

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## DETECTION AND MOLECULAR CHARACTERIZATION OF A NOVEL *CRYPTOVIRUS* FROM PERSIMMON (*DIOSPYROS KAKI*)

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Two dsRNA molecules with an estimated length of 1.5 Kbp were identified and characterized from leaves of a Japanese persimmon (*Diospyros kaki*) tree, showing veinlet necrosis on both sides of the leaf blades. DOP-PCR recognized two genomic fragments of a bipartite cryptic virus, for which the name *Persimmon cryptic virus* (PeCV) is proposed. RLM-RACE led to the sequencing of a 1,510 bp contig identified as dsRNA-1 and a 1,491 bp complete segment identified as dsRNA-2. The two genomic fragments were both monocistronic and harbored conserved domains related to RNA-dependent RNA polymerase (RdRP) and capsid protein (CP) of the species associated with the *Alphacryptovirus* genus. Phylogenetic analysis of RdRP sequence showed highest amino acid identity with *Black raspberry cryptic virus* (BrCV, 63 %), *Pepper cryptic virus 2* (PCV-2, 52 %) and *-1* (PCV-1, 46 %). Whereas the CP putatively encoded by dsRNA-2 shared the highest identity with *Mulberry cryptic virus 1* (MCV-1, 49 %), PCV-2 (39 %) and PCV-1 (32 %). N-J phylogenetic analysis confirmed these relationships and delivered PeCV in a cluster with phyto-cryptoviruses belonging to genera *Alpha-* and *Betacryptovirus*, quite different from myco-cryptoviruses, gathered in genus *Partitivirus*. Virus-specific primers for RT-PCR were successfully designed inside the CP region to detect PeCV in several symptomless trees found in different orchards of Apulia (southern Italy), thus proving that infection may be fairly common and presumably latent. An antiserum (kindly provided by Dr. M. Turina, CNR-IVV, Torino, Italy) specific to the CP of family-related *Beet cryptic virus 2* (BCV-2) was profitably used for western blot detection of a 45-50 KDa band, consistent with the predicted size of the dsRNA-2 product. Furthermore, antibodies useful for ISEM observation and subsequent decoration of PeCV particles, proved to be isometric, around 30 nm in diameter, with a rounded shape lacking in fine structural details, not easily permeable by negative stain.

## **EFFICACY OF APPLE PROLIFERATION PHYTOPLASMA TRANSMISSION BY VEGETATIVE PROPAGATION TECHNIQUES AND IMPACT ON ITS EPIDEMIOLOGY**

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In Europe, the apple proliferation phytoplasma is considered as a quarantine pest (2000/29EC) and is widespread in central and southern Europe where it is associated with significant economic losses. While its natural transmission by insect vectors has been well studied, few publications concern its transmission by grafting. We studied the efficiency of the transmission of apple proliferation phytoplasma by grafting reproducing three of the main techniques for vegetative propagation of material used in nurseries. For each experiment, we collected budwoods of young trees artificially infected by a French strain of apple proliferation phytoplasma. These trees were previously controlled by PCR and displayed characteristic symptoms of the diseases during the previous growing season if material was collected during the winter time, or during the season if material was collected during the springtime and summer season. These symptoms were foliar reddening, witches broom and enlarged stipules. Collected budwoods were grafted by three different techniques: in spring 2005 by chip budding on M7 rootstock, in summer 2006 by chip budding on M7 rootstock and in winter 2007 by bench grafting on M7 rootstock. The materials grafted in 2006 and 2007 were monitored during one growing season by visual inspection and PCR tests then by visual inspection respectively until 2010 and 2011 whereas the material grafted in 2005 was monitored in the same way until 2008. The main result revealed that after one year, only material grafted by bench grafting displayed characteristic symptoms of apple proliferation. These symptoms were further confirmed by PCR testing. Moreover, only plants showing symptoms were positive by PCR tests. The results of these experiments showed the limited impact of the propagation process on the spread of the apple proliferation phytoplasmas in nurseries and in orchards.

## **SUSCEPTIBILITY OF SOME APRICOT AND PEAR CULTIVARS ON VARIOUS ROOTSTOCKS TO ‘*CANDIDATUS PHYTOPLASMA PRUNI*’ AND ‘*CA. P. PYRI*’**

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Four pear and five apricot cultivars were bud-inoculated on different rootstocks and evaluated for their host response to pear decline (PD) and European stone fruit yellows (ESFY) phytoplasmas over a period of 3 years. As pear cultivars Santa Maria, Williams, Deveci and Ankara were grafted on *Pyrus communis* OHF333 and BA29 rootstocks whereas apricot cultivars Tyrinte, Sakıt, Tokalı, Şekerpere and Hacıhaliloğlu were grafted on Myrobolan 29 C and wild apricot. In all grafting experiments each combination was replicated ten times. Visual inspection and PCR/RFLP analyses were performed at 6 months intervals. Despite no typical symptoms of PD and ESFY being observed on any combination, phytoplasma infection from the first year after plantation was confirmed by PCR/RFLP analyses. *P. communis* and OHF 333 were the most susceptible rootstocks for local pear cv. Deveci and as well as for Santa Maria and Williams cultivars. None of the cultivars, grafted on BA 29 were found phytoplasma infected in three years. For ESFY wild apricot seems to be more susceptible for all apricot cultivars compared to Myrobolan 29 C. The apricot cultivar, Tyrinte the most susceptible to ESFY irrespectively of the rootstock employed.

## **GENOME CHARACTERIZATION OF A DIVERGENT *LITTLE CHERRY VIRUS 1* ISOLATE ASSOCIATED WITH THE SHIROFUGEN STUNT DISEASE**

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Shirofugen stunt (SS, Desvignes, 1999) is a syndrome of *Prunus serrulata* cv. Shirofugen indicators grafted with some sweet (*P. avium*) or sour (*P. cerasus*) cherry sources. It is characterized by strong rosetting with dwarfed and deformed leaves, reduced vigour, gradual necrosis at the union and sometimes die-off of the indicator after a few vegetative cycles. These symptoms are reminiscent of the recently described 'Kwanzan stunting' syndrome. The causal agent of SS is currently unknown. Double-stranded RNAs purified from the V2356 (cv. Successa) German sour cherry source of SS were sequenced using pyrosequencing technology. The 15646 reads obtained were assembled into 279 contigs. Five of these contigs, totalling almost 16.9 kbp and 5332 reads (34% of sample reads) showed high Blast scores and homology to *Little cherry virus 1* (LChV1). They were further assembled manually into three supercontigs spanning the full LChV1 genome with only two small gaps (17 and 55 bases). Completion of the sequencing of the viral genome was performed using targeted PCR and primers designed from the contigs. No evidence for other viral agents could be identified in the remaining contigs or singletons. The V2356 LChV1 isolate is only about 76% identical with the reference LChV1 sequences and, in particular, with the ITMAR isolate associated with the Kwanzan stunting syndrome. It is however highly homologous with divergent LChV1 from North America, providing the first complete sequence for such divergent isolates. Although not providing a definite proof, the failure to detect any other viral agent in the V2356 SSD source strongly suggests that the divergent LChV1 isolate identified could be responsible for the SSD syndrome. The association of similar agents in other SS sources is currently under evaluation.

## DETECTION AND CHARACTERIZATION OF PHYTOPLASMAS INFECTING APPLE TREES IN POLAND AND IDENTIFICATION OF THEIR POSSIBLE VECTORS

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During 2009-2011 a survey of the 14 apple orchards and several home gardens in different regions of Poland was conducted for the presence of phytoplasmas. In 2011, to determine putative vectors of phytoplasmas, insects were captured in ten locations using yellow sticky traps or by the beat tray method. Total DNA extracted from samples of 134 apple trees was subjected to nested PCR with primer pairs P1/P7 followed by R16F2n/R16R2. Primer sets targeting 16S rRNA and ribosomal protein (rp) gene sequences *rpl22* and *rps3* of '*Candidatus* Phytoplasma mali' (apple proliferation phytoplasma group) as well as primer pairs specific to the '*Candidatus* Phytoplasma asteris' (aster yellows phytoplasma group) target genes 16Sr RNA, rp, *secY* and *tuf* genes of were also used in this study. Phytoplasmas were detected in 12 trees. 16S rDNA RFLP analysis with *RsaI*, *HhaI*, *MseI*, *SspI* allowed us to identify '*Ca. P. mali*' in eight apple trees. Based on RFLP patterns of rp gene sequences, these isolates were identified as belonging to subgroup rpX-A, type AP. PCR/RFLP results were confirmed by sequence analysis of the 16S rDNA and rp fragments. In turn, RFLP analyses on 16S rRNA, rp, *secY* and *tuf* genes showed that four apple trees were infected with phytoplasma belonging to aster yellows group, subgroup B. In ten selected orchards 368 adults of *Cacopsylla melanoneura* 108 of *C. picta* and 77 of leafhoppers were captured. Phytoplasma presence in the insects (batches of five) was analysed by nested PCR with universal primers as well as primer pairs specific for 16SrI and 16SrX groups. '*Ca. P. mali*' was identified in two out of 73 batches of *C. melanoneura* and in four out of 21 batches of *C. picta*. '*Ca. P. asteris*' was detected in a single leafhoppers' batch out of the 15 tested.

## TRANSMISSION TRIALS OF THE FIG MOSAIC DISEASE AGENT

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### Abstract

A fig tree showing leaf mosaic (FM), heavily infested by *Aceria ficus*, was found in Emilia-Romagna. A disease-inducing agent was mite-transmitted to healthy fig plants and also to a periwinkle. Based on these previous findings, further transmission experiments have been carried out. A FM-free *A. ficus* colony was established and reared on healthy fig plants. Leaf pieces with several mites were placed on leaf pieces of the diseased periwinkle. After an acquisition access period of about 30 hours, periwinkle leaf pieces, with potentially FM-infected mites, were transferred to fig seedlings. Of the several plants tested, 7 displayed mosaic symptoms. Subsequently, one of these was selected for attempting back inoculations. Adopting the same procedure, fig and periwinkle seedlings were used and those successfully inoculated resulted to be 133 and 2, respectively. FMV was detected by RT-PCR in all FM sources identified in our experiments. The virus was also detected in *A. ficus* individuals that fed on a diseased fig. Finally, electron microscopy observations revealed the presence of double membrane bodies in leaf parenchyma cells of symptomatic, and FMV-positive, fig and periwinkle plants. These new data add further support to the suggestion that FMV could be the real FM etiological agent.

### Introduction

Recently a number of different fig (*Ficus carica*)-infecting viruses have been discovered. However, only *Fig mosaic virus* (FMV), genus *Emaravirus*, appears to be strictly associated with fig mosaic (FM) disease and the intracellular quasi-spherical double-membrane-bound bodies (DMBs), reported by various authors, are now considered the particles of this novel virus (Elbeaino *et al.*, 2009).

A fig tree showing the typical leaf mosaic was identified in Emilia-Romagna (northern Italy); symptomatic leaves appeared to be also infested by the eriophyid mite *Aceria ficus*, the natural vector of the FM pathogen. This source of FM and the colonizing vector were used for initial investigations (Credi, 1998). A disease-inducing agent was mite-transmitted from fig to fig and, unexpectedly, also to a periwinkle (*Catharanthus roseus*) plant. This exhibited leaf chlorotic spotting, mosaic, yellowing and malformation of the flowers. Based on these findings, transmission experiments were extended to acquire more knowledge about the FM phenomenon.

## Materials and Methods

A FM-free *A. ficus* colony was established and reared on healthy fig plants; small portions of heavily infested leaves were placed onto wetted leaf pieces of the FM donor sources (FM-E, FM-R) and kept under moist conditions for about a 30-h period; the FM-affected leaf pieces with mite presence were then transferred to the receptor plants (Tab. 1).

Detection of fig viruses in plant samples was performed by RT-PCR using nine sets of specific primers (Tab. 2), and total RNAs, extracted with an RNeasy Plant Mini Kit (Qiagen), as templates. Analysis conditions were gathered, with a few modifications, from the specific literature (Elbeaino *et al.*, 2006, 2007, 2009, 2010; Gattoni *et al.*, 2009; Walia *et al.*, 2009; Tzanetakis *et al.*, 2010). *A. ficus* individuals were collected from a FM-affected fig seedling (FM-R) and similarly tested, following a procedure reported by Mielke-Ehret *et al.* (2010). For ultrastructural examinations, samples of symptomatic leaves were processed according to standard protocols (Martelli and Russo, 1984); thin sections were then viewed with a Philips CM 10 transmission electron microscope.

## Results and Conclusion

Leaf pieces of the symptomatic periwinkle (FM-E) infested by *A. ficus*, originally FM-free, were transferred to 115 healthy fig seedlings. Of these, 7 plants displayed leaf mosaic symptoms a month post inoculation (Tab. 1). One was then selected and employed as inoculum source (FM-R) for further back-inoculation assays. A total of 135 fig seedlings and 6 periwinkles were used as receptor plants, and those successfully inoculated by *A. ficus* resulted to be 133 and 2 (FM-H, FM-I), were successfully inoculated by *A. ficus*, respectively (Tab. 1).

Molecular analyses performed by RT-PCR showed that the FM sources used/identified in our transmission experiments contained a single infection of FMV (Tab. 2, Fig. 1); the virus was also detected in a pool of several individuals of *A. ficus* that fed on the FM-R source (Tab. 2, Fig. 2). Finally, electron microscopy observations revealed the presence of DMBs in leaf parenchyma cells of the original FM-affected tree and in both the FM-E and FM-R sources (Fig. 3).

In conclusion from our work convincing biological evidence was obtained indicating FMV as the real causal agent of FM disease. Periwinkle, the new plant host found outside of the *Ficus* genera and also the *Moracea* family, could be an important starting point for future studies.

Tab. 1 - Transmission trials of the fig mosaic (FM) disease agent by *Aceria ficus*

‘Inoculum sources of FM’ and Receptor plants <sup>(*)</sup>	Receptor plants (N°)	
	Mite-infested	Diseased <sup>(*)</sup>
‘Periwinkle (FM-E)’		
-fig seedlings	115	7 (FM-R)
‘Fig seedling (FM-R)’		
-fig seedlings	135	133
-periwinkles	6	2 (FM-H, I)

(\*) Within brackets denomination of the FM sources used and/or identified

Tab. 2 - RT-PCR assays for virus detection in the FM-affected plants used/identified in transmission experiments by *Aceria ficus*.

FM sources	Fig-infecting viruses <sup>(*)</sup>							
	FMV	FLMaV-1	FLMaV-2	FLV-1	FMMaV	AFCV-1	AFCV-2	FBV-1
Periwinkle (FM-E)	+	-	-	-	-	-	-	-
Fig seedling (FM-R)	+	-	-	-	-	-	-	-
Periwinkle (FM-H)	+	-	-	-	-	-	-	-
Periwinkle (FM-I)	+	-	-	-	-	-	-	-
Aceria ficus (fed on FM-R)								

(\*) Specific primers (denomination and reference within brackets) used for the detection of FMV = *Fig mosaic virus* (E5-s/a: Elbeaino *et al.*, 2009; EMARAVNP: Walia *et al.*, 2009), FLMaV-1 = *Fig leaf mottle-associated virus 1* (N17-s/a: Elbeaino *et al.*, 2006), FLMaV-2 = *Fig leaf mottle-associated virus 2* (F3-s/a: Elbeaino *et al.*, 2007), FLV-1 = *Fig latent virus 1* (CPtr1/2: Gattoni *et al.*, 2009), FMMaV = *Fig mild mottle-associated virus* (LM3s/a: Elbeaino *et al.*, 2010), AFCV-1 = *Arkansas fig closterovirus 1* (Tzanetakis *et al.*, 2010), AFCV-2 = *Arkansas fig closterovirus 2* (Tzanetakis *et al.*, 2010), FBV = *Fig badnavirus 1* (Tzanetakis *et al.*, 2010); + and - = positive and negative amplification, respectively.

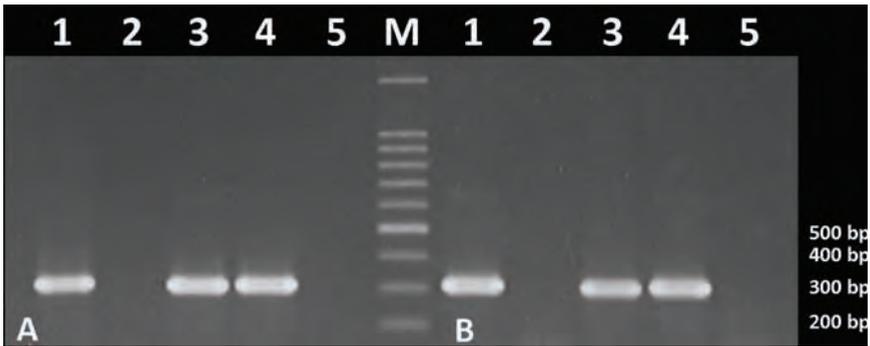


Fig. 1 - Detection of FMV by RT-PCR in FM-affected plant samples. Agarose gel showing amplification products obtained using FMV-specific primers E5-s/a (A) and EMERAV-NP (B). Lane 1, leaves from the tree naturally affected by FM. Lane 2, leaves from a healthy fig seedling. Lane 3, symptomatic leaves of the mite-infected periwinkle (FM-E). Lane 4, symptomatic leaves of a mite-infected seedling (FM-R). M = 100-bp DNA ladder (Promega).

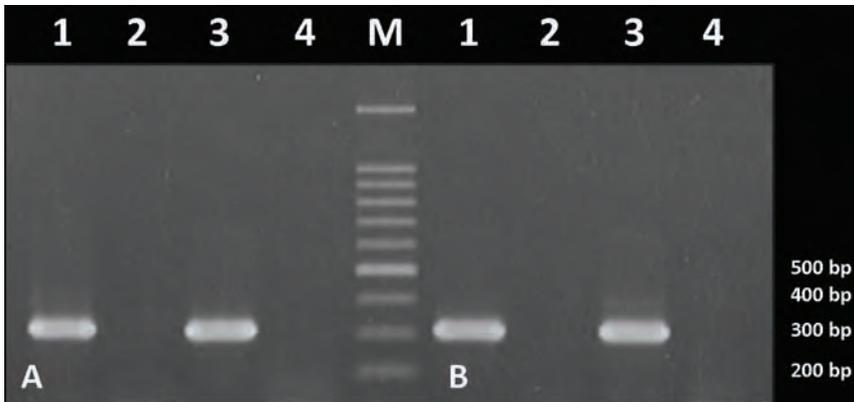


Fig. 2 - Detection of FMV by RT-PCR in a pool of *A. ficus*. Agarose gel showing amplification products obtained using FMV-specific primers E5-s/a (A) and EMERAV-NP (B). Lane 1, leaves of a virus-infected fig seedling (FM-R). Lane 2, leaves from a healthy fig plant. Lane 3 and 4, a mite pool collected from infected (FM-R) and non-infected fig plants, respectively. M = 100-bp DNA ladder (Promega).

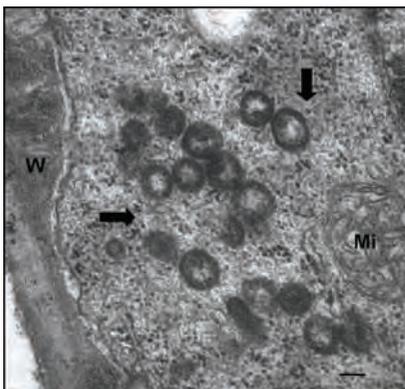


Fig. 3 - Group of DMBs (arrows), the putative FMV particles; these were observed in leaf cells of a fig tree naturally affected by FM, the periwinkle plant (FM-E source) infected with *A. ficus* individuals infesting the original FM source, and the fig seedling (FM-R source) inoculated with mites that fed on the FM-E donor source. W: cell wall; Mi: mitochondria; bar: 100 nm.

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## FIRST SURVEY ON POME FRUIT VIRUSES IN LEBANON

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In order to evaluate the incidence of virus diseases in apple, pear and quince in Lebanon, a large-scale survey was carried out in the main pome fruit-producing areas. To this aim, the most important commercial orchards and nurseries in the country were tested by a combination of biological (woody indexing and mechanical inoculation), serological (ELISA) and molecular (RT-PCR) assays in order to detect the following viruses: *Apple chlorotic leaf spot virus* (ACLSV), *Apple stem pitting virus* (ASPV), *Apple stem grooving virus* (ASGV), and *Apple mosaic virus* (ApMV). A total of 968 samples (713 apples, 175 pears and 80 quinces) were tested by DAS-ELISA, revealing that ACLSV, ASPV and ASGV were prevalent in apple trees, with an average rate of infection with at least one of the viruses corresponding to 27%. The incidence of the same viruses in pear samples was 1 % whereas no virus was detected in quince. ACLSV was the most predominant virus (16%) followed by ASPV (13 %) and ASGV (2.5%). In contrast, ApMV was not detected in any of the tested samples. RT-PCR assays performed on a fraction of 100 samples, confirmed the DAS-ELISA results, although additional positive samples were identified, showing a higher sensitivity of the former with respect to the latter detection method. The implications of our study for improving the phytosanitary status of pome fruit trees in Lebanon will be discussed.

**PARTIAL CHARACTERIZATION OF *FIG MOSAIC VIRUS* IN  
INOCULATED FIG AND PERIWINKLE PLANTS BY  
DOP-PCR**

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*Fig mosaic virus* (FMV) belongs to the recently proposed genus *Emaravirus* of the family *Bunyaviridae* and is one of the causal agents of fig mosaic disease. In this study, partial characterization of FMV in experimentally inoculated fig (DE10) and periwinkle (V19) plants by *Aceria ficus* Cotte was performed by DOP-PCR analyses. DsRNAs were isolated from leaves after first symptom appearance and cDNA was synthesized by using random primers. DOP-PCR was performed and amplicons were transformed to *Escherichia coli* DH 5 $\alpha$  after ligation of pGEM-T-Easy vector. Plasmids were selected and isolated after growing on media and sequenced. Sequence analyses were done by VectorNTI program and NCBI. The obtained partial sequences showed high similarity with Italian (98 %) and Californian (99 %) FMV isolates. Electron microscope observations confirmed double membrane bodies which are related to FMV, in palisade mesophyll cells of inoculated fig seedling.

## IDENTIFICATION AND MOLECULAR CHARACTERIZATION OF '*CANDIDATUS PHYTOPLASMA PYRI*' IN PEAR TREES FROM CENTRAL ITALY

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During a field survey carried out in early autumn, pear trees showing symptoms resembling those associated with phytoplasma presence were found in an orchard located in the Latium region (central Italy). Samples from four distinct symptomatic plants were collected and submitted to molecular detection. DNA extracts from collected samples were amplified on 16S genes using the universal primer pairs P1/P7 and R16F2n/R2 in direct and nested PCR, respectively. Amplicons of the expected size were obtained from all tested samples and the presence of '*Candidatus Phytoplasma pyri*' (16SrX-C subgroup) was detected for the first time in the Latium region by RFLP analysis of the R16F2n/R2 amplicons after digestion with *RsaI* and *SspI* restriction enzymes. In order to further investigate the genetic characteristics of the identified '*Ca. P. pyri*' isolates a preliminary characterization was performed on the *tuf* gene using a PCR/RFLP based method. All samples were then submitted to a multilocus sequence analysis on *aceF*, *pnp*, *secY* and *imp* genes. RFLP analysis of the *tuf* gene amplicons showed the presence of two distinct profiles. Out of four tested trees, three samples showed the same RFLP profile, identical and not distinguishable from those of the pear decline (PD) reference control whereas a different profile was found in the remaining sample. On the basis of multilocus sequence analyses, a high identity percentage (99-100%) was observed among the *secY* and *imp* nucleotide sequences of all tested isolates, always corresponding to '*Ca. P. pyri*'. High sequence similarity with '*Ca. P. pyri*' was also observed on *aceF* and *pnp* genes in 3 out of 4 analyzed isolates whereas the remaining sample showed the *aceF* and *pnp* genes highly identical (99%) to a '*Ca. P. prunorum*' genotype. This sample did not correspond to those showing a different RFLP profile on the *tuf* gene thus suggesting no correlation with the genomic variability observed in the investigated genes. The obtained results show that molecular variability occurs also among '*Ca. P. pyri*' isolates from the same orchard and confirm the evidences of the existence of inter-species recombination in '*Ca. P. pyri*', already reported by other author. This is the first report of possible recombinant genotype in '*Ca. P. pyri*' Italian isolates.

**DETECTION AND MOLECULAR CHARACTERIZATION  
OF *CHERRY GREEN RING MOTTLE VIRUS* (CGRMV) AND  
*CHERRY NECROTIC RUSTY MOTTLE VIRUS* (CNRMV) IN  
SWEET CHERRY IN CHILE**

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In Chile, sweet cherry represent one of the most valuable crops with approximately 13,140 ha of plantations in 2010, and the country is the main producer of cherries in the southern hemisphere. In one orchard of the Maule region (south of Santiago), plants of the cv. Bing, with and without brown angular necrotic spots, areas of dead tissue, and shot holes of the leaves, were sampled during spring and summer time. Samples were analyzed by RT-PCR with specific primers for the detection of *Prunus necrotic ringspot virus* (PNRSV), *Prune dwarf virus* (PDV), *Apple chlorotic leaf spot virus* (ACLSV), *Cherry green ring mottle virus* (CGRMV) and *Cherry necrotic rusty mottle virus* (CNRMV). All plants with symptoms tested positive for CGRMV and CNRMV. CGRMV was detected in a few plants without symptoms, while PNRSV was found in some plants with and without symptoms. Primers were designed for amplification of the gene encoding the coat protein of CNRMV and CGRMV. The amplicons obtained were purified and cloned. Putative recombinant clones were analysed by colony-PCR using primers to vector sequences flanking the polylinker. Amplicons obtained from three colonies per cloned fragment were sequenced in both directions. The sequence analysis allowed us to establish a phenetic relationship between Chilean CNRMV isolates with another detected in India, and Chilean CGRMV isolates with those found in USA, Canada, Poland, Lebanon and Italy. It is known that CGRMV does not cause symptoms in cherry varieties of commercial interest, therefore results suggest that CNRMV is the cause of the observed symptoms, which may be more severe when the plant is simultaneously infected by PNRSV. This is the first molecular characterization of Chilean isolates of CGRMV and CNRMV.

## MOLECULAR VARIABILITY IN THE COAT PROTEIN GENE OF THE DIFFERENT ISOLATES OF *APPLE MOSAIC VIRUS*

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Hop (*Humulus lupulus* L.) is without doubt one of the most intensively cultivated perennial crop in the Czech Republic representing a very important agricultural export commodity with a traditional place among the global hop producers. It is a host of several plant viruses with *Apple mosaic virus* (ApMV) identified as the predominant viral pathogen on the cultivated plants. Its infection used to have a dramatic influence on yield and quality of hop production in the past; however, thanks to the certification scheme for the production of healthy plants for planting, the ApMV has now been almost eliminated from crop production. ApMV isolates from 23 asymptomatic Czech, European and non-European hop plants were obtained from the Hop Research Institute Co., Ltd. In addition, ApMV isolates from European elder, mountain ash, hazelnut, peach and apricot were obtained and included in the study. The complete coat protein (CP) genes were amplified by using the RT-PCR. DNA amplicons were cloned and their nucleotide sequences were determined. The study objectives were to define the molecular variability of genomic information among the Czech ApMV isolates from hop and to compare them to those isolates from different areas or to those from various hosts. The identities of CPs were 95.2-100% and 91.2-100% with the nucleotide and amino acid sequences, respectively. In the phylogenetic analysis, no reasonable correlation between geographic origins of hop plants was observed. However, the host origin played a significant role in the CP heterogeneity. Together with several previously characterized ApMV isolates from the GenBank database, ApMV strains could be classified into three subgroups in both nucleotide and amino acid levels: subgroup I with ApMV isolated from almond tree, subgroup II with all pome fruit isolates and subgroup III with hop, European elder, mountain ash, hazelnut, peach, apricot, prune and *Prunus mahaleb*.

**NOVEL GENOMIC RNA SEGMENTS OF *FIG MOSAIC VIRUS*,  
A NEWLY IDENTIFIED *EMARAVIRUS* INFECTING THE  
COMMON FIG (*FICUS CARICA*)**

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Recently, *Fig mosaic virus* (FMV) was identified as the causal agent of fig mosaic disease (FMD). FMV was reported to be a four-segmented negative-strand RNA virus in the newly formed genus *Emaravirus*, whose putative members are thought to consist of four or five RNA segments. In this study, we collected fig (*Ficus carica*) leaves with typical FMD symptoms from Japan and Serbia, and detected 14 FMV isolates. In an attempt to amplify all four of the known viral RNA segments of FMV (RNA1, RNA2, RNA3, and RNA4) simultaneously, we performed RT-PCR using primers based on the conserved 13-nt stretches found at the 3' and 5' termini of FMV genomic segments. The resulting simultaneous amplification of all FMV genomic segments yielded the four previously identified segments of FMV and two novel segments from all 14 FMV isolates, and northern blot analysis was performed to characterize these two novel RNA segments. Consequently, both the sense and antisense strands of these novel RNA segments were detected in FMV-infected fig leaves, confirming that they replicate as FMV genomic segments. Sequence analysis revealed that the novel RNA segments are similar in their organization: each contains a single open reading frame on the viral complementary RNA strand flanked by untranslated regions that include the conserved, complementary terminal sequences. Moreover, the novel RNA segments showed similar molecular evolutionary patterns to those of known FMV genomic RNA segments. Our findings indicate that these newly discovered RNA segments are previously unidentified FMV genomic segments, which we have designated RNA5 and RNA6.

## **INVESTIGATIONS ON THE OCCURRENCE OF THREE LATENT APPLE VIRUSES THROUGHOUT THE YEAR AND SEQUENCE VARIABILITY OF *APPLE STEM PITTING VIRUS***

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*Apple stem pitting virus* (ASPV) is a latent virus of apple and belongs to the genus *Foveavirus*, family *Flexiviridae*. Mixed infections with *Apple chlorotic leaf spot virus* (ACLSV) and *Apple stem grooving virus* (ASGV) can cause up to 60% loss of yield. Virus RNA extractions were made from buds or leaves, phloem, and roots from four different trees at monthly intervals throughout the year followed by RT-PCR tests for evaluation of seasonal detection reliability for ASPV. In tests using published ASPV primers phloem was the most reliable tissue for detecting the virus throughout the year in 20 year old trees. Mixed infections of ASPV, ACLSV and ASGV were established by grafting onto young apple trees. These were examined monthly by RT-PCR for the three viruses, beginning one year after infection. The results showed that April was the preferable month for virus detection in leaves while analyses in the summer months were less reliable. To investigate further variability of ASPV, the complete sequences of European ASPV isolates PB 66 and Han were obtained and compared. Isolates PB 66 and Han showed 80% and 74% nucleotide sequence identity, respectively, with PA 66. The ASPV isolates PB 66 and Han showed 75% nucleotide sequence identity. An alignment of the coat protein gene revealed deletion events associated with PB 66 and Han when compared to PA 66. The coat protein of ASPV Han also has an additional insertion event. The complete coat proteins were compared with database entries and a phylogenetic tree was constructed.

**DETECTION OF *CHERRY VIRUS A* AND *CHERRY GREEN RING MOTTLE VIRUS* IN GREEK SWEET CHERRY ORCHARDS**

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Sweet cherries are susceptible to a number of virus species of the family *Betaflexiviridae*, but so far the presence of such viruses has not been extensively studied in Greek orchards. During surveys conducted in spring 2007 and 2009 for the presence of betaflexiviruses in cherry plantations of northern Greece, *Cherry virus A* (CVA) and *Cherry green ring mottle virus* (CGRMV) were identified using a generic nested RT-PCR targeting a fragment of the viral RdRp, which has been developed previously in our lab. For the specific detection and study of the incidence of each of these viruses, the nested PCR step of the generic *Betaflexiviridae* assay was modified to include CVA and CGRMV specific primer pairs. Thus, new degenerate primers were designed based on those available in the databases and herein obtained RdRp sequences of both viruses and used in respective nested PCR assays. The developed methods showed a high sensitivity and broad detection range. The application of the assays for screening 86 cherry trees from various areas of northern Greece showed a rather high incidence for both CVA (19/86) and CGRMV (20/86). Sequencing of the amplified products confirmed the specificity of the assays while it further indicated the presence of variability within each of the virus species tested. To our knowledge these findings represent the first report of CVA in sweet cherry trees in Greece and call for large scale surveys for the study of both viruses in more *Prunus* species, for which currently no information exists.

## **PARTIAL CHARACTERIZATION OF A DISTINCT *LITTLE CHERRY VIRUS 1* ISOLATE REVEALS HIGH INTRASPECIES GENETIC VARIABILITY**

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*Little cherry virus 1* (LChV1) and *Little cherry virus 2* (LChV2), two members of the family *Closteroviridae*, are associated with Little cherry disease (LChD) which is distributed worldwide and has a great impact on the fruit quality of infected trees. Both viruses are characterized by high intraspecies variability with LChV1 being the most variant. During surveys conducted in 2002 for the presence of these viruses in sweet cherry orchards of northern Greece, one sample in which LChV-1 and -2 could not be detected with specific primer pairs, gave positive reaction in closterovirus infection using a generic nested RT-PCR targeting the N terminus of the viral HSP70. Sequencing and comparative analysis of the product amplified with the generic assay indicated the presence of a virus distantly related to the so far known LChV-1 isolates and therefore it was further characterized. Using combinations of LChV-1 specific and generic primers a 5900 nucleotide long fragment of the virus genome including the helicase (partial), RdRp, the small hydrophobic protein, the HSP70 homolog and the p61 (partial) genes was determined. High sequence divergence was compared to other known LChV-1 isolates ranging from 7-10% in the RdRp up to 21% in amino acids in the p61 protein. Intriguingly, the variability increased from the 5' to 3' end of the genome. Phylogenetic analysis using the N terminus of the HSP70h placed this isolate in a separate clade most closely related with a partially characterized Californian isolate. These findings demonstrate that LChV-1 variability may be even higher than previously assumed, thus putatively affecting the reliability of the currently used detection assays and necessitating further investigation.

## THE MONITORING OF OCCURRENCE OF FOUR COMMON VIRUSES IN PLUM CULTIVARS IN LATVIA

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Viral diseases of fruit trees can cause considerable losses in commercial orchards. Timely monitoring of virus infections and elimination of infected trees from the orchards is the key point in preventing the spread of viruses. In previous studies using ELISA the presence of ilarviruses in plum orchards were detected. In this study the occurrence of *Apple mosaic virus* (ApMV), *Prunus dwarfvirus* (PDV), *Prunus necrotic ring spot virus* (PNRSV) and *Plum pox virus* (PPV) were investigated and detection rate by ELISA and RT-PCR were compared. In total 654 leaf samples of different cultivars were collected from several commercial plum orchards during 2008. The samples were collected randomly from trees without symptoms. PNRSV and PDV were detected by RT-PCR in 30.7% and 16.4% of the samples examined, respectively. The results indicated that these two viruses are widespread in comparison to ApMV and PPV, which were detected in 1.8% and 1.5% of the samples, respectively. Many plum trees were infected with two viruses. The most frequently (11 %) detected mixed infection was PDV+PNRSV. ApMV in combination with PDV was detected in 0.5% of the tested samples and ApMV with PNRSV was detected in 1.2% of the samples. Mixed infection of three viruses was detected only in a few samples. The PPV-D strain was detected by strain specific primers in all positive samples. In total, 52.4% of the samples tested by RT-PCR were negative. No specific relation between particular virus and host cultivars was observed. As expected, comparative analyses showed that RT-PCR was more sensitive than ELISA. With RT-PCR the number of positive samples for PNRSV and PDV was almost two times higher than obtained by ELISA. Therefore methods based on nucleic acid analysis are now widely used and considered more reliable for diagnosis of fruit tree viruses than ELISA.

## A NEW *TRICHOVIRUS* ISOLATED FROM PEACH TREES IN MEXICO

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A survey of peach samples from different Mexican regions by multiplex RT-PCR with the capability of detecting and identifying eight stone fruit tree viruses revealed the presence of an unexpected amplicon of 277 nucleotides which electrophoretic mobility did not match with any of the analyzed viruses. The cloning and further analysis of the nucleotide sequence revealed identity percentages of 83% and 77% with the coat protein gene of *Cherry mottle leaf virus* and *Peach mosaic virus*, respectively. The use of specific primers aimed at conserving regions of tricoviruses permitted the amplification of the 3' end 2,205 nucleotides. Blast analysis of the new nucleotide sequence rendered the highest percentage of identity for *Peach mosaic virus* (DQ117579) with 68.2%. The Blast analysis of the amino acids sequences corresponding to the end of the movement protein (409 residues), the coat protein and the nucleic acid binding protein, revealed that the highest percentages of identity/similarity corresponded to *Cherry mottle leaf virus* (66%/82%), *Peach mosaic virus* (78%/90%) and *Cherry mottle leaf virus* (57%/75%), respectively. All together, these results suggest that the new viral sequence characterized in Mexican peach orchards corresponds to a new *Trichovirus*. The complete nucleotide sequence of this potential new tricovirus is under progress.

## DETECTION OF PHYTOPLASMAS IN CIRUELO TREE (*CYRTOCARPA EDULIS*) AND IN SHARPSHOOTER *HOMALODISCA LITURATA* IN THE STATE OF BAJA CALIFORNIA SUR, MEXICO

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### Abstract

Using scanning electron microscopy (SEM) and PCR techniques the association of phytoplasmas with yellow-type disease in ciruelo in the Mexican state of Baja California Sur was recently investigated. The presence of phytoplasmas in the salivary glands of sharpshooter *Homalodisca liturata* was also confirmed using the both techniques, providing for the first time the scientific information about a possible participation of this insect in phytoplasma transmission.



Fig. 1 - Symptomatic (arrow) and asymptomatic ciruelo trees in CIBNOR experimental field.



Fig. 2 - Leaf deformations, strong anthocyan and yellowing on ciruelo shoot.



Fig. 3 - Adult *H. liturata* feeding on tree.

## Introduction

Ciruelo (*Cyrtocarpa edulis* var. *edulis*), a commonly known as 'ciruelo del monte', is a characteristic wild fruit tree in Baja California Peninsula, very popular among local inhabitants for its edible fruits with a slightly acid taste.

During phytopathological surveys in 2008-2011 in the main agricultural areas of state of Baja California Sur (BCS) some yellow type symptoms were observed in ciruelo trees throughout the state: interveinal and total chlorosis with yellowing of apical and internodal leaves reduced in size, with deformed leaf lamina and marginal anthocyan, shortened internodes and leafstalks, proliferation of shoots and dried flowers (Fig. 1-2). Such symptoms could be related to phytoplasma infection. When symptoms developed rapidly, some shoots dried over 3-4 months.

A high incidence of smoke tree sharpshooters *Homalodisca liturata* (Homoptera: Cicadellidae) was noted in ciruelo during monitoring of insects with sticky yellow traps (Fig. 3).

## Materials and Methods

Samples of apical leaflets, midribs, leafstalks, shoots and flowers from ciruelo plants exhibiting different symptoms of presumed phytoplasma infection and from asymptomatic samples were taken from trees growing in El Comitan in CIBNOR experimental fields and from ciruelo plots in San Pedro, BCS, during June-September of 2008 and 2011. Samples were processed for SEM analysis by an originally modified technique (Lebsky and Poghosyan, 2007) and examined with a scanning electron microscope (S-3000N, Hitachi, Japan) at different accelerating voltage (5-20 kV).

For molecular analysis total DNA was extracted from samples by the modified method of Zhang *et al.*, (1998). Nested PCR performed using R16MF2/R16MR1 was followed by R16F2N/R16R2. PCR products were subjected to 1% agarose gel electrophoresis and visualized by staining with ethidium bromide and UV illumination.

The specimens prepared from adults of *H. liturata* salivary glands were processed by the same techniques.

## Results and Discussion

Phytoplasmas were detected in phloem tissue of all analyzed samples from symptomatic and some asymptomatic plants (Fig. 4, A-B). They were observed as spherical cells, separate or clustered, ranging in size from 400 to 1800 nm, both in sieve tubes and a phloem parenchyma. The concentration of pathogen in phloem tissue depended on disease stage and severity, but generally was high, with affinity to host cell membrane (Fig. 4B) and vacuole (Fig. 4A). Some phytoplasmas were detected in the process of binary fusion, or with buds (Fig. 4A). The fibril structure of cytoplasm was distinguishable in host cells in some cases (Fig. 4A). Phytoplasma cells were detected also in salivary glands of sharpshooters, with an average size of 1000 nm (Fig. 5, A-B).

Nested PCR analysis of processed samples from ciruelo and *H. liturata* salivary glands revealed a 1,250 bp amplified product, thus supporting the presence of phytoplasmas.

A sharpshooter *H. liturata* was recently monitored in various woody crops and ornamentals of BCS, feeding yucca, ciruelo, legumes and other plants, in which phytoplasmas were detected by SEM (Poghosyan and Lebsky, 2010). Unlike leafhoppers, the most known phytoplasma vectors worldwide, the members of the sharpshooters subfamily of *Cicadellidae* are not well known to transmit this pathogen; there is the only one reported sharpshooter phytoplasma vector from this group, *Graphocephala confluens* (Weintraub and Beanland, 2006). Sharpshooters are mainly known as xylem feeders, transmitting xylem inhabiting bacterial pathogen *Xylella fastidiosa* (Hopkins, 1989). However, they also feed from other tissues (Katsar *et al.*, 2007).

Our work provides additional data supporting the possible participation of *H. liturata* in phytoplasma transmission in agroecosystems of BCS, and a role of ciruelo as a wild host plant for phytoplasmas. Work is in progress for the molecular identification of phytoplasmas in ciruelo and *H. liturata*.

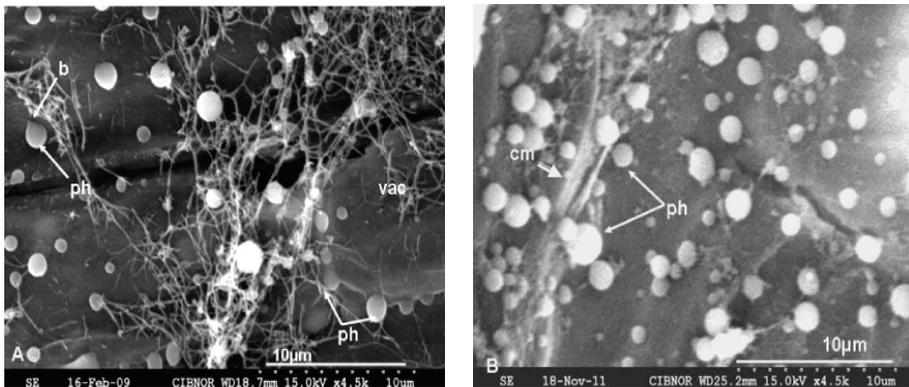


Fig. 4 - SEM micrographs of phytoplasmas in leaf midrib of asymptomatic (A) and symptomatic (B) plants. Arrows indicate: ph-phytoplasmas; b-bud; cm-cell membrane. vac-vacuole.

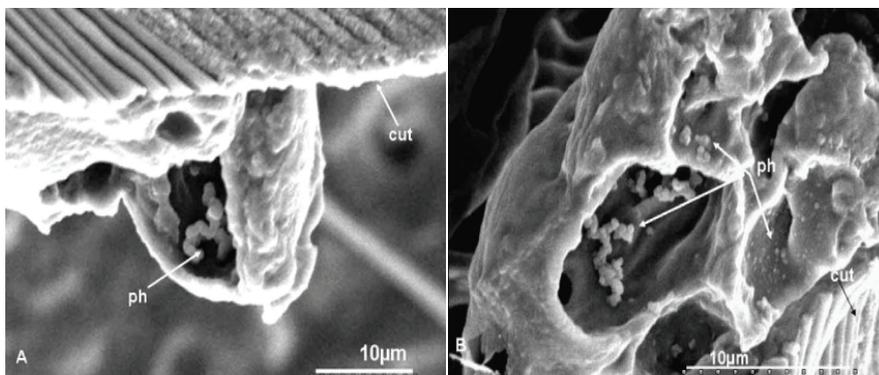


Fig. 5 - (A, B). Phytoplasmas in salivary gland of *H. liturata*. ph-phytoplasmas; cut-insect cuticle.

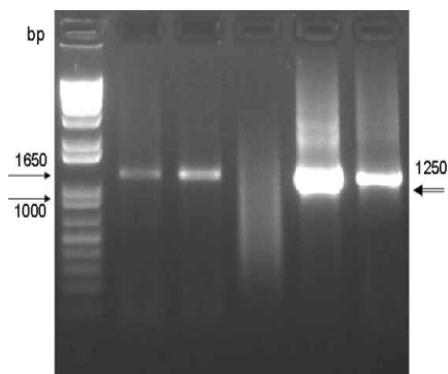


Fig. 6 - Agarose gel electrophoresis of nested-PCR amplifications of 1,250 bp fragments. Lines: 1- 1kb ladder; 2-3: *H. liturata*; 4- negative control; 5- positive control; 6- plant sample.

## **A SIMPLE METHOD FOR PHYTOPLASMAS TRANSMISSION BY GRAFTING**

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Phytoplasmas are insect-transmitted bacterial pathogens infecting a diverse range of plants, and causing economic losses in numerous crops, ornamentals, and fruit trees. They can be transmitted from original diseased host plants and diseased periwinkle to healthy periwinkle by grafting, insect vectors and use of dodder plants. This research details a simple method for transmitting phytoplasmas affecting periwinkle to healthy periwinkle, a method that also has potential for use in transmitting from other plants into periwinkle. Twelve phytoplasmas including Chrysanthemum yellows, *Rehmannia glutinosa*, ribes in vinca, strawberry green petal, crotalaria saltiana phyllody, sweet potato little leaf, vinca coconut phyllody, plum leptonecrosis, elm yellows, potato witches' broom, brinjal little leaf and apple proliferation were transmitted from diseased periwinkle to healthy periwinkle by inserting 1.5 cm long sections of diseased stem explants into the stem of the healthy plant directly. All phytoplasmas were successfully transmitted to plants and showed typical disease symptoms 6-8 weeks after transmission. Nested PCR and *secA* gene sequencing were used to confirm that transmission had occurred.

## GENETIC DIVERSITY OF *APPLE CHLOROTIC LEAF SPOT VIRUS (ACLSV)* IN FRUIT CROPS

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ACLSV is one of the most common fruit tree viruses worldwide. It infects pome and most of the stone fruits. Based on biological and serological properties, two distinct ACLSV strains (apple and peach) have been identified. Also comparisons of complete nucleotide sequences of several ACLSV isolates from plum, apple, cherry and peach have shown high sequence variability. Such high genetic variability usually arises from mutations and recombination during the replication of the virus genome. Recombination in natural populations has been reported for fruit crop viruses from the family *Betaflexiviridae* the putative recombination events in the CP gene and phylogenetical relationships as well as molecular variability of 25 Latvian ACLSV isolates from several apple (*Malus × domestica* Borkh.), pear (*Pyrus communis* L.) and plum (*Prunus domestica* L.) cultivars were analysed. Phylogenetical analyses were done in MEGA5 and recombination analysis was done using the RDP 3.44. Data regarding pair wise comparison displayed high genetic variability in CP gene among ACLSV isolates. The sequence similarity among full CP of twenty-five ACLSV Latvian isolates was 87.98 – 99.48% at nucleotide and 84.46 – 99.48% at an amino acid level. The minimal homology between nucleotide and amino acid sequences retrieved from GenBank was 66% and 69.4%, respectively. Also several putative recombination signals were detected in the CP encoding gene at N' terminal, where MP overlap the CP gene, which is described as the hypervariable region. Despite wide molecular variability, the estimates of evolutionary divergence between host groups showed that divergence between *Malus*, *Pyrus* and *Prunus* isolates is low and also phylogenetic tree lost grouping by hostplants. Only some *Prunus* isolates clustered separately from other isolates. This could mean, that for some plum isolates have different evolutionary pathways from other fruit isolates and due to intensive agriculture practices the virus has lost its primary host specificity.

## A SURVEY FOR VIRUSES AND VIRUS-LIKE PATHOGENS IN THE U.S. CHERRY GENETIC RESOURCES

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### Abstract

A survey of the U.S. plant genetic resources of cherry was conducted during 2009 and 2010. Two hundred fifty-one samples were collected from cherry trees of different species with different geographic origins in four collections. The samples were tested by RT-PCR for 14 viruses, dot-blot hybridization for two viroids and phytoplasma by nested PCR. Eleven viruses, *American plum line pattern virus*, *Apple chlorotic leaf spot virus*, *Cherry green ring mottle virus* (CGRMV), *Cherry necrotic rusty mottle virus* (CNRMV), *Cherry virus A* (CVA), *Little cherry virus 1*, *Little cherry virus 2* (LChV-2), *Plum bark necrosis stem pitting associated virus*, *Prune dwarf virus*, *Prunus necrotic ringspot virus* (PRNSV) and *Tomato ringspot virus*, were detected. Hop stunt viroid was detected only in one wild cherry (*Prunus undulata*). With the exception of the National Clonal Germplasm Repository at Davis, California, the virus infection in three other collections was very common. A majority of the plants in the three locations were infected by at least two viruses, with the most common viruses as CVA (85.1%), LChV-2 (48.8%) and PRNSV (18.4%). RT-PCR products from some isolates of four target viruses were cloned and sequenced. Sequence analyses showed various degree of genetic diversity among the isolates of different viruses. Low genetic diversity suggested that the high incidence of CVA in two locations might be due to propagation of clonal scions or rootstocks from infected mother trees.

### Introduction

The genus *Prunus* includes sweet, sour and flowering cherries, which are important fruit and ornamental trees. The USDA National Plant Germplasm System (NPGS) curates cherry genetic resources in Davis, California and Geneva, New York. The U.S. National Arboretum in Washington, D.C. maintains flowering cherry accessions, and some flowering cherry trees on the National Mall represent the earliest introductions of the ornamental cherry trees into the United States. These resources are preserved to ensure genetic diversity in these species and are used by industry as

nursery stock to propagate trees and in breeding programs to produce new varieties worldwide.

Cherry trees are vegetatively propagated and have long life spans, and thus are prone to infection by plant viruses. Many viruses and virus-like pathogens have been reported to infect cherries, and some of them cause important diseases (Hadidi *et al.*, 2011). The most common viruses and viroids infecting cherry include ACLSV, CGRMV, CNRMV, PDV, PNRSV and PLMVd (Isogai *et al.*, 2004; Mandic *et al.*, 2007; Caglayan *et al.*, 2008; Gümüs *et al.*, 2008; Matic *et al.*, 2008; Myrta and Savino, 2008; Rouag *et al.*, 2009). These pathogens may be transferred from tree to tree through grafting and persist in infected trees, allowing unintentional distribution of the pathogens. They affect the quality and yield of cherry trees, resulting in economic loss (Hadidi *et al.*, 2011).

Many cherry accessions of the NPGS and the U.S. Arboretum were collected from different geographic regions over past century before implementation of the quarantine program. Therefore, they probably harbor viral and virus-like pathogen/pathogens which may be distributed through the nursery and breeding programs to orchards and fields. Understanding the virus status in these resources will provide a baseline to manage these viral pathogens. This study aimed to gather data on health status and incidence of viruses and viroids in the USDA cherry resource collections. A survey was conducted in 2009 and 2010 in the four locations. A total of 251 cherry trees of different species with different geographic origins were collected and tested by conventional RT-PCR for 14 viruses, dot-blot hybridization for two viroids and phytoplasma by nested PCR. The pathogens included in the survey were *American plum line pattern virus* (APLPV), *Apple chlorotic leaf spot virus* (ACLSV), *Apple mosaic virus* (ApMV), *Asian prunus virus* (APV), *Cherry green ring mottle virus* (CGRMV), *Cherry necrotic rusty mottle virus* (CNRMV), *Cherry rasp leaf virus* (CRLV), *Cherry virus A* (CVA), *Little cherry virus 1* (LChV-1), *Little cherry virus 2* (LChV-2), *Plum bark necrosis stem pitting associated virus* (PBNSPaV), *Prune dwarf virus* (PDV), *Prunus necrotic ringspot virus* (PNRSV), *Tomato ringspot virus* (ToRSV), *Hop stunt viroid* (HSVd), *Peach latent mosaic viroid* (PLMVd) and phytoplasma. With the exception of the Davis Repository, the virus infection in three other collections was very common. The results also indicated that genetic variation occurs among isolates of LChV-2, CGRMV and CNRMV.

## Materials and Methods

**Sample collection and preparation:** Plant samples (leaves, buds, stems, petioles and/or bark) were collected from several positions of each tree in later winter or early spring of 2009 and 2010. Fifty samples from the Davis Repository included 18 species (*P. armeniaca*, *P. avium*, *P. bokhariensis*, *P. cerasus*, *P. cerasifera*, *P. fruticosa*, *P. mahaleb*, *P. mira*, *P. mume*, *P. pseudocerasus*, *P. serrulata*, *P. simonii*, *P. spinosa*, *P. tenella*, *P. tomentosa*, *P. undulate*, *P. virginiana* and *P. yedoensis*) from 23 countries in Africa, America, Asia, Australia and Europe; 91 samples from the Geneva Repository included two species (*P. cerasus* and *P. fruticosa*) from 10 countries in America, Asia

and Europe; 51 samples from the U.S. Arboretum were flowering cherries of 8 species (*P. cyclamina*, *P. laurocerasus*, *P. mume*, *P. nipponica*, *P. sargentii*, *P. serrulata*, *P. subhirtella* and *P. yedoensis*) and several hybrids; 60 samples from the National Mall were mainly *P. yedoensis* from Japan and *P. serrulata* of unknown sources. Majority of these trees did not show any obvious disease symptoms. Total nucleic acids were extracted from pooled tissues (bark and buds from dormant budwood or bark, leaves and petiole from shoots) of each sample as described by Li *et al.* (2008).

**Detection of pathogens:** Fourteen viruses infecting *Prunus* spp. were tested by conventional RT-PCR. Primer pairs used in this study are listed in Tab. 1. Primers for RT-PCR amplification of ACLSV, ApMV, APV and LChV-2 were designed based on alignment of sequences of each pathogen available in the GenBank. These primers were selected to bind to conserved regions of target pathogens since multiple sequences of different isolates were available. Primers available in the literature were used for APLPV (Li *et al.*, 2008), CGRMV/CNRMV (Li and Mock, 2005), CRLV (James and Upton, 2005), CVA (Li *et al.*, 2008), LChV-1 (Bajet *et al.*, 2008), PBNPaV (Al Rwahnih *et al.*, 2007), PDV (Li *et al.*, 2008), PNRSV (Marssat *et al.*, 2008) and ToRSV (Li *et al.*, 2011). The primers for the CRLV detection were slightly modified. RT-PCR for detection of these viruses was conducted using the SuperScript™ III One-Step RT-PCR System with Platinum® Taq High Fidelity (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instruction. The RT-PCR amplification was performed in a 20- $\mu$ l reaction containing 1  $\mu$ l of total nucleic acid extract, 1.0  $\mu$ l of each primer (10  $\mu$ M), 10  $\mu$ l of 2 $\times$  Reaction Mix, 0.4  $\mu$ l of Enzyme Mix and 6.6  $\mu$ l of water. The general thermal cycling conditions for RT-PCR were 1 cycle of 50°C for 30 min and 94°C for 2 min, 35 cycles of 94°C for 30 s, 60°C for 30 s and 68°C for 40 s and one final extension at 68°C for 5 min. The PCR products were electrophoresed on 1% agarose gels in TAE buffer and visualized by staining with ethidium bromide (0.5  $\mu$ g/ml) under UV illumination. The dot-blot hybridization using a digoxigenin-labeled polyprobe was conducted as described by Lin *et al.* (2011). Nested PCR for detection of phytoplasmas were performed using HotStar Taq DNA Polymerase (Qiagen) as described by Lee *et al.* (1993) with modification (Li *et al.*, 2008).

**Cloning, sequencing and sequence analysis:** RT-PCR amplicons from four commonly found viruses, CGRMV, CNRMV, CVA and LChV-2, were purified by precipitation with 1/10 volume of 5M ammonium acetate, pH 5.0 and one volume of isopropanol and subsequently cloned into pGEMO-T Easy vector (Promega, Madison, WI, USA) according to the manufacturer's instructions. The nucleotide (nt) sequence was determined for either on one direction or both directions of at least three clones for any given fragment of each isolate using an automated DNA sequencer (MacrogenUSA, Rockville, MD, USA). Sequences of clones from each amplicon were assembled using CAP3 Sequence Assembly Program (PBIL). The virus origin of each contig was confirmed by BLAST SEARCH of the GenBank Databases. Phylogenetic analyses of different isolates of each virus obtained in this study and from the GenBank Databases were conducted using MEGA 5.05, and neighbor-joining trees were constructed using 1000 bootstrap replicates.

## Results and Discussion

**Pathogen prevalence:** A total of 251 samples from different cherry species were analyzed by the conventional RT-PCR using virus-specific primers (Tab. 1) and the dot-blot hybridization (Lin *et al.*, 2011). Infection of each pathogen was confirmed by direct sequencing of the amplicons from selected samples. With the exception of

Tab. 1 - Primers used in this study

Primer*	Sequence (5'-3')	Target	Product size	Reference
ACLSV7	AAGATCGCAGAAGGGGATATTC	ALCSV	1180 bp	This study
ACLSV8b	TGTTCCGGAAGATGGYCTCCAG			
APLPV1	GACGAGGCTGATGAAGGGAA	APLPV	501 bp	Li <i>et al.</i> , 2008
APLPV2	ACCCACAGAAGGACCTACCA			
ApMV11	TGGAAACTCAACCGAGCAGATAC	ApMV	903 bp	This study
ApMV14	ACCAACTAACTTCTCAGG			
APV2F	CCTAGAACCCTGGAGAGGCCT	APV	749 bp	This study
APV2R	GTACGGTCTGTCAAGCGAACAC			
CGRMV1	CCTCATTACATAGCTTAGGTTT	CGRMV, CNRMV	958 bp, 959 bp	Li & Mock, 2005
CGRMV2	ACTTTAGCTTCGCCCGGTG			
CGRMV3	GACATACTGGGACCAAG	CGRMV	632bp	This study
CGRMV4	TGAATTTGGCCAGTCTGCC			
FAVR1-7Fm	TGACTTTCCCAAGGATGAGAAG	CRLV	452 bp	James and Upton, 2005
FAVR1-8Rm	CACTGTGACATACCATAGATCC			
CVA1	GTGGCATCAACTAGCACTAT	CVA	876 bp	This study
CVA2	TCAGCTGCCTCAGCTTGGC			
LChV1-3EC	GCCTAGAGGCCCTTTTATTTTTATATATGC	LChV-1	276 bp	Bajet <i>et al.</i> , 2008
LChV1-16659	GTATAGAATTCACTGCAAGTG			
LChV2-1	TTCAATATGAGCAGTGTCTTAAC	LChV-2	781 bp	This study
LChV2-2	ACTCGCTTTGTGACATACCACTC			
PBNSPaVf	TACCGAAGAGGGTTTGGATG	PBNSPaV	400 bp	Al Rwahnih <i>et al.</i> , 2007
PBNSPaVr	AGTGGCACCACAGTCTTCT			
PDV1	GGAAAACCTACTGCCCTTC	PDV	540 bp	Li <i>et al.</i> , 2008
PDV4	ATCGAGTGTGGAGTACTGAGT			
PNRSV10F	CTTGAAGGACCAACCGAG	PNRSV	348 bp	Massart <i>et al.</i> 2008
PNRSV10R	ATCTGCTAACCGAGGTAAG			
ToRSV5	AGGTAGGACGCYATTGTTCCAGG	ToRSV	433 bp	Li <i>et al.</i> , 2011
ToRSV6	AGTCTCAACTTAACATACCCTAC			

\* The first primer of each primer pair is forward primer, and another is reverse primer.

Tab. 2 - Numbers and rates of plants infected by different target viruses\*

	CVA	LChV-2	CGRMV	CNRMV	APLPV	PNRSV	PDV	PBNSPaV	ToRSV	ACLSV	LChV-1
Geneva	88/91 (97%)	15/91 (16%)	3/91 (3%)	17/91 (19%)	5/91 (5%)	5/91 (5%)	2/91 (2%)	0/91	0/91	0/91	2/91 (2%)
Davis	2/50 (4%)	0/50	2/50 (4%)	1/50 (2%)	1/50 (2%)	5/50 (10%)	2/50 (4%)	2/50 (4%)	nd	0/50	0/50 (2%)
Arboretum	28/50 (56%)	36/50 (72%)	1/50 (2%)	0/50	2/50 (4%)	9/50 (18%)	1/55 (2%)	4/55 (7%)	1/55 (2%)	4/55 (7%)	0/55
National	56/60 (93%)	36/60 (60%)	6/60 (10%)	1/60 (2%)	9/60 (15%)	0/60	2/60 (3%)	6/60 (10%)	3/60 (5%)	0/60	0/60

\*ApMV, APV and CRLV were not detected.

Tab. 3 - Virus infection status

	No virus	One virus	Two viruses	Three viruses	Four viruses
Davis	36	12	2	0	0
Geneva	1	35	32	20	3
Arboretum	7	13	18	9	3
National Mall	4	36	10	10	0

the Davis Repository, virus infections were common (Tab. 2). Whereas the overall infection rate was 82%, infection rates were 99% for the Geneva Repository, 97% for the National Mall, 86% for the Arboretum and 28% for the Davis Repository, respectively. A majority of the plants tested from the Geneva Repository and National Mall were infected with at least two target viruses, whereas most of the cherry accessions in the Davis Repository were not infected with any target viruses (Tab. 3). Of mix infected trees, majority were infected with CVA and/or LChV-2

with other viruses. The higher phytosanitary status in the Davis Repository might result from earlier implementation of the certified programs in California, confirming the importance of quarantine and certificate programs for the fruit tree production. Eleven viruses were detected (Tab. 2). Among them, CVA (70%) and LChV-2 (35%) were the most frequently found viruses. With the exception of the Davis Repository, the two viruses were prevalent in other locations. Low incidences of APLPV (7%), CGRMV (5%) and PDV (3%) were observed in all four locations. The six other viruses (ACLSV, CNRMV, LChV-1, PNRSV, PBNSPaV and ToRSV) were found in low incidences in some locations. The dot blot hybridization disclosed that only one of wild cherry (*P. undulata*) from India was infected by HSVd, indicating the viroid infection was not common. Infection of ApMV, APV, CRLV, PLMVd and phytoplasma was not detected. This survey was conducted in the late winter and early spring, and low incidence of LChV-1 and viroid, therefore, could also be a result of lower pathogen titer in these seasons. A similar survey was recently conducted for *Prunus* spp. in the Davis Repository recently, and a similar result was reported for the cherry species (Osman *et al.*, 2012). The presence and incidence of different viruses were slightly different from our results. This difference might be the result of different cherry accessions, sampling season and/or primer specificity. Most pathogens detected in this survey probably represent the original infections of the pathogens since many cherry accessions in these locations were collected from different regions worldwide before the implementation of the USDA quarantine programs. However, the high incidences of CVA and LChV-2 in some locations suggested that the viruses probably originated from scions or rootstock propagated from limited infected source plants.

**Host species and geographic origins of viruses:** The collections at the National Arboretum, Davis and Geneva Repositories are composed of different species from many countries, whereas two-thirds (40) of cherry trees in the National Mall are *P. yedoensis* ‘Yashino’ from Japan. A total of 22 *Prunus* species were included in this survey, and most were *P. armeniaca* (14), *P. cerasus* (78), *P. fruticosa* (16), *P. mume* (18) and *P. yedoensis* (51). The host species and geographic origins of each virus detected in this study are listed in Tab. 4. HSVd was detected in a wild cherry from India (data not shown). The results show that some pathogens such as PNRSV and APLPV infect many cherry species and are widely distributed worldwide, whereas others such as ACLSV and ToRSV occur in very low incidence. The results also show that many cherry species are hosts of CVA and LChV-2, and they might be widely distributed. These pathogens can be distributed to commercial orchards by propagating and breeding materials since all these pathogens are transmitted through propagative materials.

**Genetic diversity of four common viruses:** To analyze genetic variation of four common viruses, nucleotide sequences of 84 CVA, 19 CGRMV, 11 CNRMV and 41 LChV-2 isolates were obtained from RT-PCR amplicons by direct sequence or RT-PCR cloning and sequencing. Phylogenetic analysis based on the 726-bp sequences of the CVA isolates with genetic divergence larger than 1.0% indicates that these isolates were grouped into two clusters, with 31 of 34 National Mall isolates in cluster I and 36 of 37 Geneva isolates in Cluster II (Fig. 1). Genetic divergence of the CVA isolates within each cluster was up to 9%, 13-22% between two cluster.

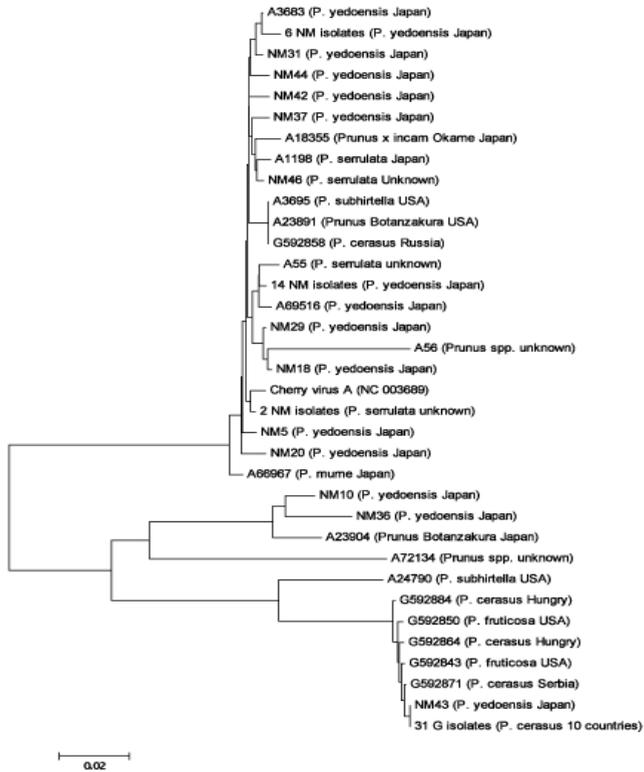
Therefore, the sources of the virus in the two locations are probably different. The divergences among 31 isolates from the National Mall in cluster I were 0-2%, whereas the sequences of 31 Geneva isolates in cluster II were identical, supporting limited infection sources in these two locations. The Arboretum isolates, on the other hand, were different from each other in the sequences, indicating that they might come with the infected cherry accessions from different sources. The LChV-2 isolates could be divided into two clusters, but genetic divergences within each cluster were higher (up to 5%) than that of the CVA clusters (Fig. 2). All Arboretum isolates were grouped in cluster II, whereas many isolates from the National Mall were in cluster I. Therefore, the sources of LChV-2 were also limited. Both CGRMV and CNRMV were detected in the National Mall and Geneva Repository as well as the Davis Repository (Fig. 3). Genetic divergence ranged from 1-11% among the CGRMV isolates and 1-15% among CNRMV isolates. A Thailand isolate of *P. mume* shared only 72-74% identities with other isolates of the two viruses, indicating that it might be a distinct virus. These results indicate that genetic variation occurs among the isolates of CGRMV and CNRMV from different species and geographic origins. This survey provides essential basic information for the future management strategy of cherry genetic resources in the United States. The survey revealed that CVA and LChV-2 are the most common viruses in three of four locations, which might have been introduced from limited scions or rootstock. Therefore, testing rootstock for the newly described pathogens is as important as quarantine testing of imported plant materials in the collection of genetic resources of fruit trees. To prevent a further distribution of the pathogens, a pathogen elimination therapy using cryopreservation (Towill *et al.*, 2004) or meristem tip culture is also recommended. A preliminary evaluation of the sour cherry accessions regenerated from 12-year cryopreservation showed that CVA, CGRMV, CNRMV and PNRSV were detected in the regenerated seedlings from infected mother plants, whereas LChV-2 was absent, indicating the method is only effective in elimination of certain virus (our unpublished data).

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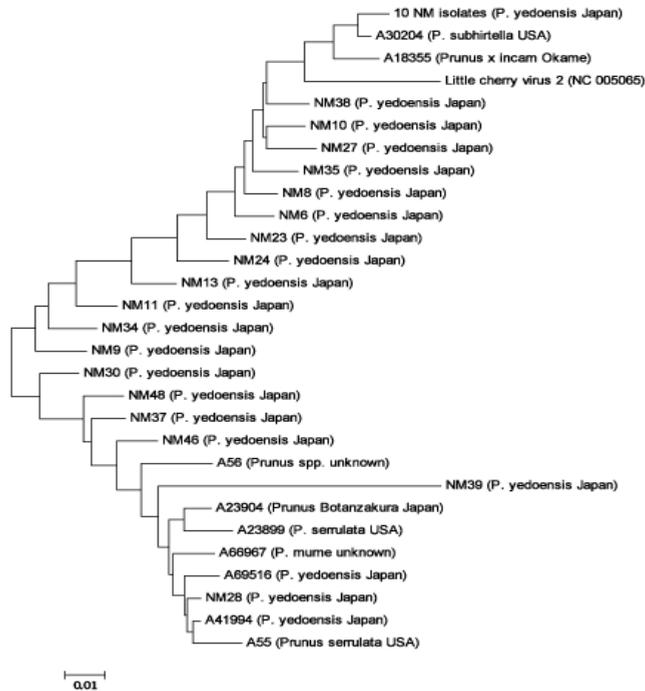
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#### **Acknowledgements**

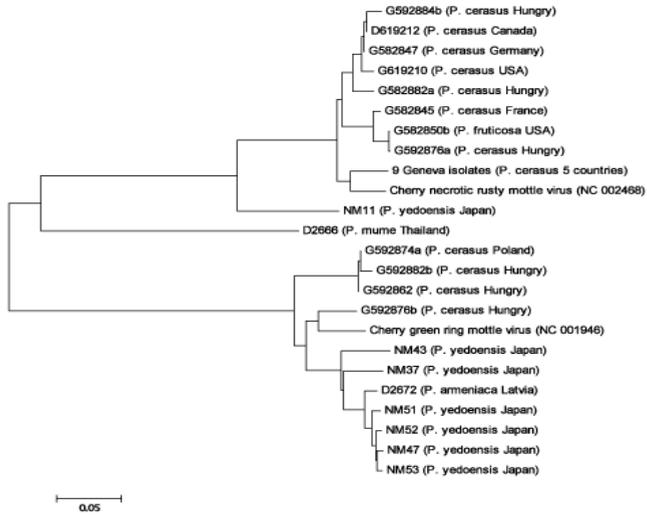
We thank Whitney Hymes, Liming Lin, Eun Ju Cheong, Sam Grinstead and Donglin Xu for technical assistance.



**Fig. 1** - A neighbor-joining tree based on the 726-bp sequences obtained from 84 isolates of *Cherry virus A* (CVA). The isolates with genetic divergence less than 1% were combined as a variant for analysis. NM represents the isolates from the National Mall, and A represents the isolates from the National Arboretum. The type species of CVA was used as reference strain. Bootstrap analysis was applied using 1000 bootstrap replicates, and the scale bar represents a genetic distance of 0.02.



**Fig. 2.** A neighbor-joining tree based on the 621-bp sequences obtained from 41 isolates of *Little cherry virus 2* (LChV-2). The isolates with genetic divergence less than 1% were combined as a variant for analysis. NM represents the isolates from the National Mall, and A the isolates from the National Arboretum. The type species of LChV-2 was used as reference strain. Bootstrap analysis was applied using 1000 bootstrap replicates, and the scale bar represents a genetic distance of 0.01.



**Fig. 3.** A neighbor-joining tree based on the sequences obtained from isolates of *Cherry green ring mottle virus* and *Cherry necrotic rusty mottle virus*. The isolates with genetic divergence less than 1% were combined as a variant for analysis. NM represents the isolates from the National Mall; A represents the isolates from the National Arboretum, and D represents the isolates from Davis. The type species of the two viruses were used as reference viruses. Bootstrap analysis was applied using 1000 bootstrap replicates, and the scale bar represents a genetic distance of 0.05.

Tab. 4. Hosts and geographic origins of the viruses

Virus	Host species	Geographic origin
ACLSV	<i>Prunus x incam</i> 'Okame'	Japan
APLPV	<i>P. armeniaca</i>	India, Japan, Turkmenistan, Unknown
	<i>P. bokhariensis</i>	
	<i>P. cerasus</i>	
	<i>P. serrulata</i>	
	<i>P. yedoensis</i>	
	<i>Prunus</i> 'Snow Fountain'	
CGRMV	<i>Prunus</i> 'Amayadori'	Hungry, Japan, Poland, Thailand
	<i>P. cerasus</i>	
	<i>P. fruticosa</i>	
	<i>P. mume</i>	
CNRMV	<i>P. yedoensis</i>	Canada, France, Hungry, Japan, Poland, Romania, Russia, USA
	<i>P. cerasus</i>	
	<i>P. pensylvanica</i>	
CVA	<i>P. yedoensis</i>	France, Hungry, Italy, Japan, Poland, Romania, Russia, USA, Unknown
	<i>P. armeniaca</i>	
	<i>P. cerasus</i>	
	<i>P. cyclamina</i>	
	<i>P. laurocerasus</i>	
	<i>P. mume</i>	
	<i>P. nipponica</i>	
	<i>P. sargentii</i>	
	<i>P. serrulata</i>	
	<i>P. subhirtella</i>	
	<i>P. yedoensis</i>	
	<i>Prunus</i> 'Snow Fountain'	
	<i>Prunus</i> 'Amayadori'	
	<i>Prunus x incam</i> 'Okame'	
<i>Prunus x sieboldii</i>		
LChV-1	<i>P. cerasus</i>	Russia
LChV-2	<i>P. armeniaca</i>	Hungry, Italy, Japan, Romania, Russia, USA, Unknown
	<i>P. cerasus</i>	
	<i>P. cyclamina</i>	
	<i>P. laurocerasus</i>	
	<i>P. mume</i>	
	<i>P. nipponica</i>	
	<i>P. sargentii</i>	
	<i>P. serrulata</i>	
	<i>P. subhirtella</i>	
	<i>P. yedoensis</i>	
	<i>Prunus x incam</i> 'Okame'	
	<i>Prunus x sieboldii</i>	
	<i>Prunus x 'Snofozam'</i>	
	PBNSPaV	
<i>P. cerasifera</i>		
<i>P. mume</i>		
<i>P. serrulata</i>		
<i>P. yedoensis</i>		
PDV	<i>P. cerasus</i>	France, Japan, Poland, Russia, Unknown
	<i>P. mume</i>	
	<i>P. subhirtella</i>	
	<i>P. yedoensis</i>	
	<i>Prunus x incam</i> 'Okame'	
PNRSV	<i>P. bokhariensis</i>	Australia, France, Germany, Hungry, India, Italy, Japan, Morocco, Romania, Russia, Serbia, Unknown
	<i>P. cerasifera</i>	
	<i>P. cerasus</i>	
	<i>P. fruticosa</i>	
	<i>P. pseudocerasus</i>	
	<i>P. serrulata</i>	
	<i>P. takesimensis</i>	
	<i>P. yedoensis</i>	
	<i>Prunus x incam</i> 'Okame'	
	<i>Prunus x sieboldii</i>	
ToRSV	<i>P. yedoensis</i>	Japan

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**MONITORING OF PSYLLID SPECIES  
(HEMIPTERA, PSYLLOIDES)  
IN CONIFEROUS PLANTS IN POLAND**

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During 2010-2011, monitoring of the psyllid fauna species overwintering in coniferous plants was carried out at the Botanical Garden in Powsin, Warsaw, Poland. Specimens were collected by using the beating tray method in *Pinus sylvestris* Laponica, *P. tabuliformis*, *Picea pungens* Tomek trees 'Candidatus Phytoplasma pini' affected and in phytoplasma free *Pinus nigra*, *P. mugo* and *Abies alba* trees. In the samples collected in 2011 four species of psyllids were identified. Of these species, three, *Aphalara polygoni* Förster, *Aphalara exilis* (Weber & Mohr) and *Cacopsylla melanoneura* (Förster) were already known to occur in Poland whereas *Cacopsylla affinis* (Löw) was recorded for the first time. Similarly Triozidae was represented by four species. Among these, *Heterotrioza remota* (Förster) was predominant while *Trioza urticae* (L.), *Trioza rhamni* (Schrank) and *Trioza nigricornis* (Förster) were sampled sporadically. Species known as vectors of phytoplasmas belonging to 16SrX phytoplasma group were studied as a priority. Infection with phytoplasma was studied by amplification of DNA using polymerase chain reaction with subsequent RFLP analysis in selected individual. Phytoplasma strain belonging to 16SrX phytoplasma group were detected in two specimens of *C. affinis* and in one individuals of *T. rhamni* collected from *P. sylvestris* Laponica.

## **FIRST REPORT OF MOLECULAR IDENTIFICATION OF '*CANDIDATUS PHYTOPLASMA PYRI*' IN PEAR TREES IN BELGIUM**

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Pear decline (PD) is a quarantine organism for the European Union that has been assigned to the Candidatus taxon, '*Candidatus Phytoplasma pyri*'. In October 2010, nearly 140 samples of leaves showing early yellow or red discoloration were collected in pear production orchards in north-west Belgium. DNA was extracted from 0.5 g of leaf midribs using a simplified extraction method. Samples were then tested for phytoplasma presence by PCR using the universal primer pair fU5/rU3, all yielding PCR products of expected size. BLAST analysis of a sequenced amplicon revealed 100% identity with '*Ca. P. pyri*' strains (16SrX group). About 15% of the collected samples were infected. The results of the characterization of these isolates are presented. To our knowledge this is the first report of '*Ca. P. pyri*' in orchard pear trees in Belgium, confirmed by molecular tests.

## IDENTIFICATION OF *PEACH LATENT MOSAIC VIROID* (PLMVd) IN MONTENEGRO

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*Peach latent mosaic viroid* (PLMVd) and *Hop stunt viroid* (HSVd) isolates can cause latent infections, but may also induce dapple fruit symptoms, fruit and stone deformations, fruit cracking, discoloured spots, mosaic, extreme chlorosis, delayed bud break, flowering and ripening, enhanced susceptibility to biotic and abiotic stress, bud necrosis, and tree decline. 13 samples of 12-year-old peach trees (cv. Elegant Lady) were collected in the most important peach-growing area of Montenegro in the vicinity of Podgorica. Some of the trees showed mild or severe mosaic, chlorotic rings, and fruit deformations, but they were also infected with *Plum pox virus*. RT-PCR analyses using RF-43/RF-44 primers for PLMVd resulted in positive amplification of an 348 bp product in nine peach samples, while RT-PCR results with primers VP-19/VP-20 for HSVd were negative for all samples. Seven PCR products of PLMVd were successfully cloned and sequenced. Consensus sequences were deposited in the GenBank under accession numbers JF927892–JF927898. Sequence analyses revealed that PLMVd isolates from Montenegro shared 92.6 to 97.9% identity with each other, 94 to 98% identity with the PLMVd isolate G sequence (EF591868) and 91.8 to 94.4% identity with PLMVd sequence M83545. Since the spread of PLMVd infections can pose a threat to Montenegrin fruit production, additional studies are required to further evaluate the incidence of the pathogen in other locations and other hosts in Montenegro.

## **AN AYPICAL ALBANIAN ISOLATE OF *PLUM POX VIRUS* COULD BE THE PROGENITOR OF THE MARCUS STRAIN**

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In the framework of the efforts supported by the European FP7 SharCo project, PPV isolate AL-11pl, found in a naturally infected plum (*Prunus domestica*) in Çërravë, Eastern Albania, was submitted to molecular analysis. RNA was extracted from infected leaves by silica capture and used for cDNA synthesis by random primers and M-MLV RT (Invitrogen). cDNA was amplified by *Taq* DNA polymerase (Fermentas) in several fragments of about 1200bp on average. Purified PCR products were directly sequenced on both strands using the respective PCR primers. Alternatively, some PCR products were ligated into the pSC-A vector (Stratagene) and recombinant plasmids were sequenced. The complete genomic sequence of the AL-11pl was assembled from a set of overlapping PCR fragments. AL-11pl genome consists of 9786 nt, excluding the 3' terminal poly (A) tail. The organization is identical to previously sequenced PPV isolates. A start codon (AUG) was identified at positions 147–149, and an amber stop codon at positions 9567–9569, resulting in a single ORF consisting of 9420 nt. Sequence comparisons indicate that the closest isolate is Turkey (Ab-Tk), with a divergence of 6.5%. Divergence with PPV-M is 7.7% (PPV-PS) to 7.9% (PPV-SK68) while other strains are more distantly related: 13.8% (Rec, PPV-BOR3), 15.5% (PPV-D isolates), 20.3% (PPV-EA), 21.2% (PPV-W) and 21.6% (PPV-C). Molecular characterization of AL-11pl shows a high divergence with PPV-M in 1-2688 region, high divergence with PPV-Tk in 1-1584 region, but high homology with PPV-Tk in 1584-2688 region. This result demonstrates that AL-11pl has the expected features for an PPV-M ancestor in evolutionary scenarios described by Glasa and Candresse (2005). AL-11pl can thus be considered as a putative further strain of PPV, for which the name PPV-An (Ancestor) is proposed. This research leading to these results has received funding from the European Community's Seventh Framework Programme (FP7/2007-2013) under Grant Agreement N°204429, SharCo project

## EPIDEMIOLOGY OF *PLUM POX VIRUS*-T AND -M ISOLATES IN STONE FRUIT ORCHARDS IN TURKEY

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Although *Plum pox virus* (PPV) was introduced in Turkey 44 years ago, the virus is present in a rather limited number of trees. Our recent results on PPV epidemiology show that PPV was introduced rapidly in new PPV-free regions. The epidemiology of PPV was studied in previously (Aegean-Izmir) and the more recently infested (Mediterranean-Antakya) regions from 2009 to 2011. Aphid populations were monitored from the first week of April to the end of June by the sticky-plant method. Aphids collected from plum and apricot trees were tested individually by squash real-time RT-PCR. The highest aphid populations were observed at the end of May in both regions but the aphid species landing on the plants were different. The most abundant aphid species caught were *Myzus persicae* (20.15 %), *Hyalopterus pruni* (18.64 %) and *Aphis craccivora* (9.04 %) in Izmir and *Aphis gossypii* (20.55 %), *Aphis spiraeicola* (19.38 %) and *Hyalopterus pruni* (13.36 %) in Antakya. Among the aphid species caught in Izmir, the percentage of PPV-viruliferous aphids were 40, 34, 25 and 9 in *M. persicae*, *H. pruni*, *A. gossypii* and *A. craccivora* aphid species, respectively. However, in Antakya infection rates were 41, 25, 21, 18 and 16 %, in *M. persicae*, *A. spiraeicola*, *A. gossypii*, *H. pruni* and *A. craccivora*, respectively. This is the first report on the epidemiology of PPV in two different ecological regions of Turkey.

## **PLUM POX VIRUS W APPEARS TO BE THE MOST VARIABLE STRAIN OF THE SEVEN RECOGNIZED STRAINS OF THE VIRUS**

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### **Abstract**

Recently 14 new isolates of PPV-W were identified in Russia. Phylogenetic analysis of the (C-ter) NIB-(N-ter) CP region of these 14 isolates, plus the published sequences of PPV-W isolates from Canada, Latvia, and the Ukraine, revealed 7 distinct clades supported by high bootstrap values. Though genetically diverse all 14 new Russian PPV-W isolates were detected by the strain specific primers 3174-SP-F3/3174-SP-R1. Eight of these isolates were selected for CP analysis. Analysis of the CP of the 8 new isolates plus the CP sequences of 5 PPV-W isolates available in GenBank revealed CP sizes of 330 to 331 aa residues. Identity among PPV-W isolates ranged from 93 - 99% nt identity, and 94 - 99% aa homology. Genetic diversity among PPV-W isolates was greater than that of isolates of any other strain of PPV, with  $n \geq 7$  CP sequences available. The aa residue valine (V) was identified in the + 1 position of the heptapeptide NIa protease cleavage site at the NIB/CP junction of an isolate. This is the first report of V occupying the + 1 position of the NIa cleavage site of any isolate or species in the genus *Potyvirus*.

### **Introduction**

*Plum pox virus* (PPV) is a positive sense single-stranded RNA virus, approximately 10 kb in size, and a member of the genus *Potyvirus*, family *Potyviridae* (Reichmann *et al.*, 1989; Shukla *et al.*, 1991; Garcia *et al.*, 1994). Genetic diversity among isolates of PPV has resulted in the development and recognition of at least seven strains of the virus; M, D, C, EA, Rec, W, and T (James and Glasa, 2006; Ulubas Serce *et al.*, 2009). The RNA-dependent RNA polymerase, responsible for the replication of RNA viruses, lacks proof reading ability which results in genetic variation or mutations among virus progeny (Garcia-Arenal *et al.*, 2001). Members of the genus *Potyvirus* are also prone to recombination events (Bousalem *et al.*, 2000; Glasa *et al.*, 2004), which contribute to genetic diversity. The recognized strains of PPV-Differ in

traits such as: aphid transmissibility, disease symptoms, host range, and geographic distribution. Genetic differences unique to members of each strain have allowed the development of strain-specific nucleic acid-based assays, and also strain-specific protein-based (serological) assays. Due to this variability in biology, strain identification is important for effective control and management of this dangerous virus.

Genetic diversity and biological differences are found also among isolates of the same strain. Two distinct serological subclusters of PPV M isolates were described by Myrta *et al.* (2001), and differences in aphid transmissibility of isolates of PPV strain D were identified by Schneider *et al.* (2011). Also, Sheveleva *et al.* (2012) identified *Prunus tomentosa* as a natural host for PPV-W isolate RD4, while Mavrodieva *et al.* (2012) found that *P. tomentosa* was not a host for PPV-W isolate 44189. In analysing and comparing the coat protein (CP) sequences of isolates of various strains of PPV, Matic *et al.* (2011) found that PPV-Rec isolates and PPV-EA were among the most genetically diverse strains. In this study evidence obtained using recently identified isolates shows that isolates of PPV strain W may be the most genetically diverse compared to isolates of the 7 strains of PPV recognized to date.

## Materials and Methods

**Virus isolates:** Eight new Russian PPV-W isolates from a range of natural hosts were selected for CP analysis. These included: isolates STNB-1, STNB-2, P2, and P3 in the host *Prunus domestica* (plum); isolate Pd2 in *P. spinosa* (blackthorn); isolates 1410-1, and 1410-7 in *P. nigra* (Canadian plum); and isolate RD4 in *P. tomentosa* (downy cherry).

**Total RNA extraction, cloning, sequencing, and sequence analysis:** Total RNA was extracted using Qiagen's RNeasy Plant RNA kit, following the manufacturer's instructions. Just prior to elution, the RNeasy spin columns were shipped to the Sidney Lab where total RNA was eluted with PCR-grade MilliQ H<sub>2</sub>O. A region in the 3'-terminus of the virus, encompassing the CP, was amplified using the primer PPV-4CPF (5'-ATACTTGAGTGGGACAGATCAATTG-3') as forward primer, and either an oligo(dT) primer or PPV-4CPR (5'-GAGAAAAGGATGCTAACAGGAATC-3') as the reverse primer (Matic *et al.*, 2011). The cDNA was gel purified using Qiagen's MinElute gel extraction kit, and cloned using Invitrogen's TOPO TA Cloning kit, following the manufacturer's instructions. A minimum of three independently generated clones were sequenced bi-directionally. DNA sequencing was carried out at the Nucleic Acid Protein Service Unit, UBC, Vancouver, BC, using Applied Biosystems 3730 DNA Analyzer. Sequence analyses were performed using Clone Manager, Professional Edition 1994-2010, Scientific & Educational Software, Cary, NC, USA. Phylogenetic analyses were carried out using CLUSTAL X (v1.83).

## Results

The CPs of all 8 Russian isolates were sequenced and found to be 990 – 993 nt in size, with deduced aa residues of 330 – 331 aa, respectively. Most isolates had CPs consisting of 993 nt, except isolates P3 (host *P. domestica*), and Pd2 (host *P. spinosa*), indicating an indel event among these isolates. Interestingly, in phylogenetic analyses based on the CP nt sequences P3 grouped more closely to PD2 than to other isolates infecting *P. domestica* such as STNB-1, STNB-2, and P2 (data not shown).

Sequence alignments and analyses were carried out on a total of 13 PPV-W isolates including: the 8 new Russian isolates, and 5 full length CP sequences of PPV-W isolates deposited in the GenBank. The accession numbers of GenBank isolates included: AY912055.1 (W3174), HQ670748.1 (LV-145bt), HQ670746.1 (LV-141pl), HQ326086\_MCW (1410), and JN596110 (UKR 44189). Analyses revealed a range of similarity among PPV-W isolates (n = 13) of 93 – 99% identity of the nt sequences, and 94 – 99% homology for the deduced aa residues (Tab. 1). A much narrower range of diversity was observed for isolates of other strains of PPV. The strains showing the least amount of genetic diversity among isolates were: PPV-D (n = 50) displaying 98-99% nt identity and 96-99 aa homology, and PPV-M (n = 28) showing 98-99% nt identity, and 99-100% aa homology (Tab. 1), with strains having 7 or more full CP sequences available.

Tab. 1 - Percentage identity of the coat protein sequences of isolates of various strains of *Plum pox virus*

Strain	No. Isolates	% ID, NT	% ID, AA
W	13	93-99	94-99
D	50	98-99	96-99
M	28	98-99	99-100
Rec	19	95-99	95-100
EA	16	95-99	95-99
C	7	97-99	94-99
T	1 & Part.	?	?

All 8 PPV-W isolates possess the DAG motif: at CP aa position 11-13 in the case of isolates P2, P3, Pd2; and at aa position 12 – 14 in the case of isolates 1410-1, 1410-7, RD4, STNB-1, STNB2. The latter isolates appear to have an additional aa residue, likely valine (V).

Interestingly, the CP of PPV-W isolate 1410-1 has a valine (V) as the first amino acid of the CP. Two clones of 1410-1 (a & b) are indicated in Fig. 1, and multiple clones were used to confirm this sequence. Alanine (A) was identified as the first aa residue of the CP of all other strain W isolates with the appropriate sequence available, as well as isolates of all other

strains of PPV (Fig. 1 & Tab. 2), except two C type isolates deposited in the Genbank with the accession numbers HQ849517 (BY101) and HQ840518 (BY181). The C type isolates BY101 and BY181 have threonine (T) as the first amino acid residue of the CP. The first aa residue of the CP forms part of the heptapeptide NIa protease

cleavage site, with isolate 1410-1 having the variant (3v) heptapeptide NIVMHQ/V, instead of NIVMHQ/A (Tab. 2, Fig. 1). Isolates BY101 and BY181 deposited in the GenBank have the variant (4v) heptapeptide NIVVHQ/T, instead of NIVVHQ/A (Tab. 2). There appear to be 4 heptapeptide sequence motifs (1 – 4, Tab. 2) associated with the 7 recognized strains, with isolates of W and C possessing variants of their respective motifs, with A or T, respectively, in the +1 position of the heptapeptide cleavage site. The most conserved aa residues of the heptapeptide motif associated with the NIa protease cleavage site at the NIb/CP junction of PPV is: NXVXHQ/A, with conserved residues at positions - 6, - 4, - 2, - 1, and + 1 (Tab. 2, underlined).

Strain	NIb/CP Cleavage Site
D	1 - NIVVHQ/A
EA	1 - NIVVHQ/A
M	2 - NIVIHQ/A
Rec	2 - NIVIHQ/A
<u>W</u>	3 - NIVMHQ/A (3v-NIVMHQ/V)
<u>C</u>	4 - NIVVHQ/A (4v-NIVVHQ/T)
T	4 - NIVVHQ/A
<b>Consensus</b>	<u><b>NXVXHQ/A</b></u>

Tab. 2 - The heptapeptide sequences of the NIa protease cleavage site at the junction of the NIb and coat protein of various strains of *Plum pox virus*, showing four (1 – 4) formats associated with the site, the consensus sequence, and the variants 3v and 4v associated with isolates of strains W and C, respectively

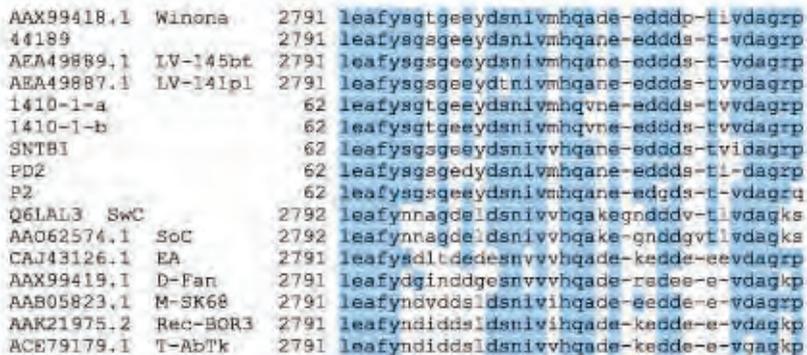


Fig. 1 - Amino acid sequence alignment at the junction of the NIb and coat protein regions of isolates representing the seven recognized strains of PPV, and showing valine (V) instead of alanine (A) at the + 1 position of the heptapeptide protease site of *Plum pox virus* W isolate 1410-1. Interestingly, in this figure the strain T isolate has GAG instead of the DAG motif shown to be essential for aphid transmission.

## Discussion

The genetic diversity found among isolates of PPV-W appears to be greater than the genetic diversity observed among isolates of all other strains of PPV recognized to date. In the analysis of the full CP sequence of 13 isolates of PPV-W, the range of nt and aa variability (1 - 7% and 1 - 6%, respectively) was among the highest seen for any strain of PPV. Isolates of PPV-W also display evidence of recombination (Myrta *et al.*, 2006; Glasa *et al.*, 2011; and Mavrodieva *et al.*, 2012); and they have a wide natural host range that include *Prunus domestica* (plum), *P. spinosa* (blackthorn), *P. nigra* (Canadian plum), and *P. tomentosa* (downy cherry) (Sheveleva *et al.*, 2012). There are even differences in host range among isolates, where Sheveleva *et al.* (2012) identified *Prunus tomentosa* as a natural host for PPV-W isolate RD4, while Mavrodieva *et al.* (2012) found that *P. tomentosa* was not a host for PPV-W isolate 44189.

Recognizing and understanding the scope of genetic diversity of any pathogen is important since this affects its biological diversity, and also our ability to reliably detect and identify the pathogen. The monoclonal antibody (MAb) 5C1 was developed against PPV-W isolate 3174, and used in a variety of assays for reliable detection of that isolate (Croft *et al.*, 2008). This MAb, however, was not effective for the detection of a range of Russian PPV-W isolates (Sheveleva *et al.*, 2012) due to genetic/protein diversity in the epitope region. The MAb EA24 is recognized as a PPV strain EA-specific antibody, but it failed to recognize the EA isolate APR 50 which has a single amino acid substitution in the epitope region that inhibited binding of MAb EA24 (Matic *et al.*, 2011). Also, a single nucleotide change in the Canadian PPV-D isolate 48-922 resulted in a change of GTAC to GTGC in the *RsaI* restriction site used for distinguishing D and M isolates, which made it difficult to determine the strain of the isolate (James and Glasa, 2006).

The PPV genome is bicistronic with two open reading frames (ORFs): a large ORF encoding a polyprotein of approximately 355.5 kDa in size (Garcia *et al.*, 1994; Shukla *et al.*, 1991), and a small overlapping open reading frame called PIPO (Pretty Interesting *Potyvirus* ORF), expressed as a fusion product of approximately 25 kDa in size (Chung *et al.*, 2008).

The large polyprotein is cleaved post translation into the mature proteins. The virus CP is cleaved at its N-terminus by the NIa protease which targets a heptapeptide recognition site (Garcia *et al.*, 1992). The conserved motif NXVXHQ/A is associated with the NIb/CP junction NIa protease heptapeptide site of PPV. This encompasses a conserved sequence motif (Val-Xaa-His-Gln↓) described by Kang *et al.* (2001). The NIa protease cleaves at several positions (identified as A, B, C, D, E, F, and V) along the polyprotein (Garcia *et al.*, 1992; Yoon *et al.*, 2000), all of which are defined by heptapeptide sequences. Cleavage occurs between the amino acids occupying the -1 and +1 positions of the heptapeptide site. In analysing the NIa protease cleavage site associated with various members of the genus *Potyvirus*, in no case was valine (V) identified at the +1 position of any of these cleavage sites (Garcia *et al.*, 1992; Yoon *et al.*, 2000).

Threonine (T) is present at the +1 position of the heptapeptide sequence of the CI/6K2 junction of PPV (Garcia *et al.*, 1992), and is found in the +1 position of at least two NIa protease sites of *Turnip mosaic virus* (Yoon *et al.*, 2000). It means therefore that finding T in the +1 position of a NIA protease cleavage site is known to occur, though unusual for isolates of PPV.

Alanine is usually the first amino acid residue of the CP of PPV. Alanine and valine are similar amino acids, both having non-polar or aliphatic R-groups. They may therefore be interchangeable. Garcia *et al.* (1992) indicated that changes in conserved residues in the heptapeptide region may influence cleavage efficiency. The effects of the substitution of valine (or threonine) for alanine in this position are unknown at this time. Threonine (T) is a non-aromatic amino acid with a hydroxyl R-group, but is known already to be present in the +1 position of NIa protease cleavage sites. In reviewing the published literature, PPV-W isolate 1410-1 is the first isolate of PPV-Described with valine (V) in that position, and it is the first case described in any potyvirus species where V is located in the +1 position of a NIa protease cleavage site.

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## PRELIMINARY EVALUATION OF THE COMPETITIVENESS OF PPV-REC AND PPV-D UNDER FIELD CONDITIONS

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PPV-D, PPV-M and PPV-Rec are the three main strains of *Plum pox virus* (PPV), the most detrimental viral pathogen of stone fruits (*Prunus* sp.). While different experimental results have reported that PPV-M is more aggressive than PPV-D, limited information is available on the competitiveness of PPV-Rec relatively to PPV-D or PPV-M. To provide this information, we monitored spread of PPV-Rec and PPV-D spreading in an experimental plum orchard (577 trees) located in Bistrita, Romania. The dynamics of the spread of the two strains was monitored during four vegetative periods (2008-2011). The sanitary status of the trees was assessed visually and by ELISA testing each year. In 2008, the strain werestatus of each PPV isolate was determined by IC-RT-PCR using strain specific primers located in the (Cter) CP, (Cter) N1b – (Nter) CP and the 6K1-CIP coding regions. From 2009 to 2011, strain typing was performed for every new disease case and for all single strain infections detected in the previous years, in order to check for possible over infection with other strain. Disease incidence increased from 57 % (328/577) in 2008 to 72 % (414/577) in 2011. From a total of 328 infected trees in 2008, 58% of the trees were infected by PPV-Rec, 13 % by PPV-D and 29% were co-infected by PPV-D and PPV-Rec. The results on the rate of progression of PPV-D and PPV-Rec as well as the frequency of co-infections will be presented and a preliminary conclusion upon the competitiveness of PPV-Rec and PPV-D in our conditions will be discussed. This research was fouded mainly by the European Community's Seventh Framework Programme (FP7/2007-2013) under Grant Agreement n°204429, SharCo project, and also by the Romanian Research Ministry, contract 34/2009.

## COMPLETE AND PARTIAL GENOME SEQUENCES OF THE UNUSUAL *PLUM POX VIRUS* (PPV) ISOLATES FROM SOUR CHERRY IN RUSSIA SUGGEST THEIR CLASSIFICATION TO A NEW PPV STRAIN

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*Plum pox virus* (PPV), the agent responsible for sharka disease, is characterized by a wide genetic diversity and currently known PPV isolates are assigned to 7 molecularly different PPV strains. Despite substantial progress in the ability to assess and describe PPV variability, our understanding of PPV variability and of the geographical distribution of this variability is still incomplete, in particular from the geographical regions which have so far been little surveyed. Within the framework of the the European FP7 SharCo project, a set of unusual PPV isolates (RU-17sc, RU-18sc, RU-19sc, RU-20sc) were analyzed from an old sour cherry plantation grown in the cultivar collection of the Horticultural research institute (Kuibyshev region) and from sour cherry trees in a private gardens (Krasnoyarsk region) in the Samara oblast, Russia. Besides being recovered from an atypical *Prunus* host, these isolates have the additional originality of not being detected in ELISA by the universal 5B monoclonal antibody (Mab). Partial sequence analysis targeting the N1b-CP region of the genome of 4 isolates (RU-17sc, RU-18sc, RU-19sc, RU-20sc) revealed about 30% nucleotide sequence divergence with conventional PPV-M, D and Rec isolates. Phylogenetic analysis clearly assigned these isolates to a distinct cluster, most closely related to PPV-C and PPV-W. The deduced aminoacid sequence of the N-terminus of the capsid protein revealed a D94>E aminoacid substitution probably responsible for the failure to react with the 5B Mab. Complete genome sequence comparisons showed that the RU-17sc and RU-18sc isolates only 73-80.6% identity with other sequenced PPV strains. These results suggest that these unusual isolates belong to a new PPV strain able to infect cherry, for which the name PPV-CR (Cherry Russian) is proposed.

## **A LARGE SCALE STUDY OF *PLUM POX VIRUS* GENETIC DIVERSITY AND OF ITS GEOGRAPHICAL DISTRIBUTION**

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An understanding of the geographic distribution and dynamics and of the genetic variability of *Plum pox virus* (PPV) populations on a worldwide scale is a prerequisite for an efficient management and control of the Sharka disease. An international effort supported by the European FP7 SharCo project aims to provide a realistic view on the current diversity of PPV-Worldwide, by the analysis of a large number of natural field isolates, collected following a standardized sampling protocol. About 800 PPV isolates from 27 countries and the corresponding epidemiologically relevant data (information on locality, host, symptomatology...) were systematically collected. Isolates were preserved in lyophilized form in a centralized collection and partial sequence data were generated for two highly informative genome portions i.e. P3-6K1 and N1b-CP, together with the complete genome sequences of 35 epidemiologically or molecularly interesting PPV isolates. Besides providing information on the presence of particular strains in countries from where they had previously been unreported, the results obtained provide a detailed cartography of the prevalence of PPV strains in the numerous sampled countries or host plants. Analysis of the partial sequence data indicates a higher than previously expected genetic diversity within the PPV-D strain and confirms the splitting of the PPV-M strain into two subclusters. Moreover, divergent isolates were identified in the PPV-D, M, Rec, T and W strains and are currently undergoing full-length genomic sequencing. The detailed synthesis of the results obtained on these various aspects will be presented and discussed.

## ***PLUM POX VIRUS SITUATION IN EMILIA-ROMAGNA REGION (ITALY)***

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*Plum pox virus* (PPV) is the causal agent of Sharka, the most dangerous disease of stone fruit trees, reducing fruit quality and yield. In Italy, Sharka was first detected in 1973 but in the Emilia-Romagna region (northern of Italy) it appeared in 1982 in a few apricot and plum orchards. Only after 1995 did it become epidemic in peach, although since 1992 surveys to evaluate the distribution of PPV have been conducted in order to contain the epidemic diffusion. Each year, from spring until mid-summer, samples with suspected symptoms were collected from orchards in five different provinces and analysed by ELISA test using both polyclonal and monoclonal antibodies and, randomly, by molecular method (RT-PCR), to distinguish different strains. Strain characterisation showed that infected peach plants hosted PPV-M whereas apricots and plums were infected by PPV-M, PPV-D or PPV-Rec. From 1997 to 2011, 3,800 farms (more than 10,000 hectares) cultivating stone fruit (orchards and nurseries) were surveyed georeferenced, by GPS and recorded in GIS; 24,600 symptomatic samples were collected and sent to the Plant Protection Service (PPS) laboratory for official analysis. In all fields where infected plants were detected, the percentage of symptomatic plants was discriminated between below or over 10%, in order to explant individual infected plants or the entire plots respectively. The costs of compulsory phytosanitary measures were refunded to growers by the regional government, which, until 2011, consisted of 5,382,233 euros. Despite of these efforts, the infection rate increased each year by about 20% and, in 2011, 263 new PPV foci (16 on apricot, 187 on peach and 60 on plum) were discovered or confirmed and 22,297 plants were removed. PPS established 9 areas where Sharka was present and established, without the possibility of eradication, surrounded by an appropriate buffer zone, 1 km large, where systematic inspections are carried out.

## **EVALUATION OF *PLUM POX VIRUS* SENSIBILITY ON DIFFERENT STONE FRUIT VARIETIES IN EMILIA ROMAGNA REGION (ITALY)**

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Between 2003 and 2011, about 200 stone fruit varieties were inoculated by chip budding with PPV M strain, grown in a screen house and then surveyed for symptom expression, on leaves, flowers and fruits during the following season in particular 126 peach and nectarine, 45 apricot and 28 plum varieties and seedlings (from breeding programs) under evaluation were assessed in the present work. A large number of varieties showed discoloration and mottling on the young leaves in spring; furthermore all peach and nectarine selections with rosaceous flowers appeared infected by PPV- With strong symptoms on petals. Many peach and apricot cultivars and seedlings, but only one plum variety, presented fruit deformations with typical rings and mottling. Only a few accessions (2 peaches and 10 apricots) belonging to ‘ancient’ germplasm or to seedlings did not appear affected by typical symptoms on leaves and fruits, nor were positive to PPV by ELISA and Real Time RT-PCR assays. These experimental data revealed high sensibility of stone fruit germplasm to PPV-M as confirmed by the information gathered in the Emilia-Romagna region, by inspectors of the local Plant Protection Service. In particular during 15 years of orchard surveys 36 varieties of apricot, 37 of plum, 102 of nectarine and 83 of peach (both traditional and promising varieties) were infected, showing typical symptoms, as a result of the natural PPV spread in the field.

## SUSCEPTIBILITY OF DIFFERENT PRUNUS ROOTSTOCKS TO NATURAL INFECTION BY *PLUM POX VIRUS-T*

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*Plum pox virus* (PPV) in stone fruit trees in Turkey was reported in 1968 and was endemic until the 2000's, in Central Anatolia, Marmara and Aegean regions, where the PPV-T strain remains widespread. There is no detailed information available about the epidemiology and response of different rootstocks to PPV-T. Thus, susceptibility of different *Prunus* rootstocks to PPV-T was assessed under natural inoculum pressure in the Izmir-Aegean region. A wide range of rootstocks was evaluated 'Nemaguard' (*P. persica* x *P. davidiana* hybrid seedling), 'Mariana' GF8.1 (*P. cerasifera* x *P. munsoniana*), Docera 6 (*P. domestica* x *P. cerasifera*), Myrobolan 29 C (*P. cerasifera*), GF677 (*P. dulcis* x *P. persica*) and 'Garnem' (*P. dulcis* x (*P. persica* x *P. davidiana*)). The rootstock plants were individually analysed for PPV by 5B-IVIA monoclonal antibody twice (spring and autumn) in 2009, 2010 and 2011. The results demonstrated that the 'Myrobolan 29C' and 'Nemaguard' rootstocks were the most susceptible to natural PPV-T infection with the infection rate of 6,14 % and 4,66 %, respectively. Moreover, typical PPV symptoms were also observed on both rootstocks. Other assayed rootstock species showed no symptoms and were negative by DASI-ELISA. The most abundant aphid species monitored by the sticky-plant method were *Myzus persicae* and *Hyalopterus pruni* when PPV isolates obtained from naturally infected rootstocks were analysed by sequencing, all isolates were characterised as PPV-T.

**MOLECULAR INVESTIGATION ON RECOMBINANT  
*PLUM POX VIRUS*  
ISOLATES IN CENTRAL ITALY**

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In the framework of an Italian project financed by the Ministry of Agriculture a monitoring was performed to verify the presence of PPV-Rec isolates in Central Italy and to evaluate the molecular variability of these isolates, with the aim of defining the appropriate modalities of containment. Regional Plant Protection Services of Central Italy provided samples from plum and peach trees with PPV symptoms (Abruzzo, Emilia-Romagna, Latium and Marche). A multigene approach was used: three different genomic regions were analyzed to verify the molecular variability of PPV isolates in order to investigate recombinant identification: C terminal of coat protein gene, 3' terminal of N1b gene and the genomic region including P3 and 6K1 genes. Coat protein (CP) gene was analyzed using RT-PCR with primers P1/P2, followed by RFLP analysis after digestion with *RsaI* restriction enzyme. N1b gene was studied using RT-PCR with four specific primers in three different combinations mM5/mM3, mD5/mD3 and mD5/mM3. P3-6K1 genome region was analyzed by RT-PCR with primers PP3/PCI, followed by RFLP analysis after digestion with the restriction enzymes *EcoRI*, *DdeI* and *VspI*. By multigene analysis several isolates were classified as PPV-M or PPV-D strains, whereas some of the isolates coming from the Emilia-Romagna region were anomalous, as they showed an RFLP pattern typical of PPV-M strain both on CP gene and P3-6K1 genomic regions, after digestion with *RsaI* and *DdeI* enzymes, respectively. On the basis of other molecular tests on the other hand appeared to belong to the PPV-D strain. The results confirmed the presence of 'anomalous' PPV recombinant isolates in Emilia-Romagna region, but, for the moment, they seem to be confined in these areas. More approaches are needed to improve the knowledge about these isolates and to verify their spread to other Italian regions.

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## TEMPORAL ANALYSIS OF *PLUM POX VIRUS* IN CHILE

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*Plum pox virus* (PPV) has been previously reported in Chile, and its presence is related exclusively with the Dideron (D) strain. Four stone fruit commercial orchards infected by PPV-D were used to provide information concerning the temporal spread of the virus in Chile. During the spring seasons of 2008 and 2011 (from September to October) different plant tissues were sampled from a block of about 400 plants at each orchard. These materials were tested by the ELISA technique, using the Realisa Reforzado kit (REAL, Spain). In orchard A (Apricot cv. Castelbrite) the prevalence increased from 11.25% in 2008 to 17.25% in 2011, while incidence decreased gradually from 11.25% (2008) to 0.6 (2011). Orchard C (Peach cv. Summer African Gold) showed a prevalence of 12.02% (2008) to 36.06% (2011), and more or less constant values of incidence: 12.02% (2008), 11.2% (2009), 8.62 (2010), 10.44% (2011). In orchard D (Prune cv. D'Agen) we observed a prevalence of 7.0% to 28.46% in four years, and alternate values of incidence: 7.0% (2008), 1.88% (2009), 13.7% (2010), 8.89% (2011). In orchard E (Nectarine cv. Artic Snow) the prevalence values were 13.25% to 26.5%, and incidence decreased gradually from 13.25% to 0.34%. After the first year of evaluation, in orchard A the positive plants were cut, while in orchard E preventive control of aphids migration was implemented. This may explain why orchards A and E achieved the lowest incidence values at the end of four years, considering that at least one known PPV vector, *Myzus persicae*, was found in the four orchards. In addition, spatial analysis is currently being carried out to assess the spread pattern of PPV, and to identify disease clusters in each study site. In 2012 the fifth and final ELISA test will be carried out in the four orchards.

## **DYNAMICS OF SPREAD OF PPV-REC AND PPV-D IN AN EXPERIMENTAL PLUM ORCHARD**

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The three *Plum pox virus* (PPV) strains (PPV-M, PPV-D and PPV-Rec) are present in Serbian plum orchards. Although PPV-Rec is widespread in plum, little is known about its epidemic properties and competitiveness, in particular compared to PPV-D. An experimental plum orchard of 400 healthy trees variety Čačanska lepotica was set up in 2008 in order to study the dynamics of the spread and competitiveness of PPV-Rec and PPV-D isolates from artificially inoculated trees. The few *Prunus* trees situated in the surrounding area of the orchard were precisely located, sampled and tested by ELISA and IC-RT-PCR for the presence of PPV. Contaminated trees were removed when possible and all positive samples were partially sequenced. One PPV-D and one PPV-Rec isolate were selected from a collection of Serbian PPV isolates to be used as artificial inoculum in the orchard (4 trees per isolate). The selection of the isolates was based on the nucleotide differences between the sequences of the 427 bp genomic fragment in order to be able to distinguish the inoculum sources from the isolates spreading in the surrounding area of the orchard. The two selected isolates were then fully sequenced and biologically characterized (assessment of aphid transmissibility). Each year, from 2008 to 2011, all trees in the orchard were visually inspected and tested by ELISA. All positive samples were strain-typed by IC-RT-PCR method and partially sequenced. Artificially inoculated trees were found to be infected in 2009. New cases of infection were detected within the orchard, with respectively 2, 18 and 33 PPV-Rec infected trees and 1, 2 and 4 PPV-D infected trees detected from 2009 to 2011. The spread of PPV-Rec and PPV-D isolates will be analyzed in the light of the spatio-temporal and viral genomic sequence data, in order to disentangle the influence of both internal and external sources of inoculum. Preliminary results will be presented and further discussed.

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## **DETECTION OF *PLUM POX VIRUS* IN REGIONS OF BELARUS**

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In Belarus, *Plum pox virus* (PPV) was first identified in the Minsk region in 2000 on symptomatic plum trees of cultivar Nagrada Nemanskaja. In 2009 and 2010 the virus disease was monitored in plantations of stone fruits in the Minsk and Brest regions. In 2011 more than 6 hectares of stone fruits, 8 plantations, were investigated for PPV presence in three regions of Belarus (Vitebsk, Grodno and Mogilev). 73 samples were collected from symptomatic trees on the basis of visual observation. PPV-Detection was carried out by ELISA and PCR analyses. PPV-Was confirmed in 32.9% out of all tested samples with visual symptoms. The most frequently (19.2% out of all tested samples) virus was detected in samples from cherry plum cv. Kometa Kubanskaja. The presence of PPV-Was detected also in samples from plum rootstocks (OD 2-3), sour cherry cv. Vjanok and sweet cherry cv. Eput.

**EFFECT OF CO-INFECTION OF *PLUM POX VIRUS*  
AND *PRUNE DWARF VIRUS* IN *PRUNUS PERSICA*; A  
QUANTITATIVE ANALYSIS BY REAL-TIME RT-qPCR**

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The effect of co-infection of *Plum pox virus* (PPV) and *Prune dwarf virus* (PDV) were studied in *Prunus persica* (GF-305). Plants were inoculated in five types of experiments: i) with PPV-D, ii) with PDV, iii) first with PPV-D then PDV, iv) first with PDV then PPV-D and v) with PPV-D and PDV at the same time. The plants were evaluated after bud breaking based on symptoms, virus detection and quantification. PPV-D and PDV were detected by ELISA and RT-PCR. The virus titre was quantified by real-time RT-qPCR. GF-305 plants inoculated with both viruses (PPV-D and PDV) were in general more dwarf than single virus (PPV-D or PDV) infected plants. In both types of mixed infected plants (experiments iii and iv) titre of PDV was higher than PPV-D. However, the titre of PDV in these mixed infected trees was as high as the solely PDV infected trees. The same was true for the titre of PPV-D. Furthermore, PPV-D was undetectable in all tested plants co-inoculating with PPV-D and PDV at the same time. PDV was detected in some of the above mentioned trees and the virus titre was much lower than solely infected PDV trees. From our results it could be concluded that mixed infection of both viruses has synergetic effect on symptom development. However, the influence of mixed infection in virus titre (PPV-D or PDV) is unclear. Further quantitative analysis of PPV-D and PDV co-infection will confirm the possible effects of mixed infection of these viruses in fruit trees.

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## SEROLOGICAL AND MOLECULAR CHARACTERIZATION OF THE ISOLATES OF *PLUM POX VIRUS* FOUND IN LATVIA.

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A survey of *Plum pox virus* (PPV) isolates present in different countries was initiated within framework of the SharCo project financed by European Union (contract n°204429, FP7/2007-2013). Leaf samples were collected in 2009 in two orchards in Latvia, from symptomatic/ suspected and symptomless plants of 21 plum (*Prunus x domestica*), 2 myrobolan plum (*Prunus cerasifera* L.) and 3 apricot (*Prunus armeniaca* L.) trees. They were tested for the presence of *Plum pox virus* with PPV-Universal DAS-ELISA kit (AMR Lab Consultants, Spain) using monoclonal antibody 5B, as well as with DAS-ELISA using locally prepared polyclonal antibodies. Nine samples, for which positive results had been obtained in these tests, were re-tested with four monoclonal antibodies: PPV-Universal (again), D-specific and M-specific (DASI-ELISA kits purchased from AMR Lab Consultants, Spain) and (subset of D specific) MAb V/8. Three samples from one orchard showed a positive reaction with MAb Un, D and V/8; one sample from the same orchard reacted positively with MAb Un and D, but not with V/8. These isolates were preliminary classified into two different serotypes of strain D. Five samples from another orchard showed a positive reaction only with PPV - universal monoclonal antibody, and could not be positively strain typed using available antibodies. Two cDNA fragments (600 nts or more) corresponding to P3-CI junction and NIB/CP junction were obtained by IC-RT-PCR method for all isolates and sequenced. The analysis confirmed the identification of the four isolates from the first orchard as D strain/type isolates. Four out of five isolates from another orchard, showed more than 94% nucleotide sequence identity of both cDNA fragments with PPV-W3174 (syn. PPV-Winona). Based on these results, Latvian isolates named LV-140pl, LV141pl, LV-143 and LV-145pl, detected in the plum hybrids 298-70 and Tern 32-34, were classified as PPV-W strain. Further research of Glasa based on full genome analysis showed that those isolates represented 'ancestral' PPV-W.

## **PARTIAL CHARACTERISATION OF BIOLOGICAL PROPERTIES OF PPV-C ISOLATES FOUND IN BELAROUS AND ESTABLISHMENT OF *IN VITRO* CULTURES OF INFECTED L2 AND OWP-6 ROOTSTOCKS**

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A survey of *Plum pox virus* isolates present in different countries was initiated in the framework of the SharCo project financed by European Union (contract n°204429, FP7/2007-2013). Several trees of *Prunus* hybrids, used as rootstocks for cherry cultivation, were found in Belarus to be infected with *Plum pox virus*, strain PPV-C. The virus was found in ten plants (>10 years old trees) of L2 (*Prunus lanesianna*) and one plant of OWP-6 (*Prunus cerasus* x *P. padus* or *P. cepadus*). Biological properties of two PPV-C isolates: BY-101 from L2 and BY-181 from OWP-6 were studied by chip bud inoculation of several potential woody hosts and mechanical inoculation of *N. benthamiana*. All experiments were performed in contained glasshouses. The woody plants tested were: *Prunus avium* x *Prunus pseudocerasus* 'Colt' – vegetatively propagated rootstock, *Prunus avium*, 'Alkavo' - rootstock propagated from seeds, *Prunus avium*, F12/1 rootstock vegetatively propagated, *Prunus persica* GF305, and Antypka (*Prunus mahaleb*). During the one-year study we found that all plants of F12/1 rootstock grafted or chip bud inoculated with PPV-C (BY-101 and BY-181) died soon after new shoots started to develop, although for a final conclusion more trials are necessary. Neither GF305 nor *P. mahaleb* became infected with the studied PPV-C isolates; all inoculated plants developed normally, showing no symptoms and detectable presence of PPV-C in our experiment. Clear symptoms were observed on all 'Colt' and several 'Alkavo' inoculated plants. The symptoms on leaves of infected L2 and OWP-6 shoots developing from buds on 'Colt' or 'Alkavo' rootstock were also quite pronounced. It must be stressed, however, that so far the plants in question have been tested for the presence of PPV, PNRSV, PDV and ACLSV, but not for the presence of other viruses. Extensive testing is needed to confirm L2 as promising indicator plant for PPV-C. It is quite possible, however, that it may be a good host for maintaining PPV-C. *In vitro* cultures were established from plants infected with PPV-C (L2 and OWP-6 rootstocks) as well as from healthy L2 plants (to be used for maintaining PPV-C isolates and potentially as indicator plant). The presence of PPV-C was verified in May 2012 by ELISA. These cultures will be used to maintain a virus in a convenient and relatively safe manner, and they will be suitable for international exchange of reference isolates. Both PPV-C isolates from L2 and OWP-6 induced strong systemic symptoms on inoculated plants of *N. benthamiana*.

## MAPPING THE PATHOGENICITY DETERMINANTS IN THE GENOME OF *PLUM POX VIRUS*

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### Abstract

Three major strains of the *Plum pox virus* (PPV), namely PPV-D, PPV-M and PPV-Rec differ by the preference of natural host species and by other biological properties observed in artificial herbaceous hosts. To map the phenotypes reflecting virus-host interactions in the PPV genome we analysed the biological properties of prepared set of inter-strain-chimeric cDNA clones. The P1/HC-pro genomic region was responsible for the hypersensitive reaction of *Nicotiana occidentalis* and *Nicandra physaloides*. *In silico* analysis mapped this phenotype determinant to the 3'-proximal part of the P1 gene. More detailed analysis by site-directed mutagenesis showed a single amino acid in the position 138 responsible for mild (R<sup>138</sup>) or severe (W<sup>138</sup>) symptomatology in these two herbaceous host plant species.

### Introduction

*Plum pox virus* (PPV) is the most destructive virus of stone fruit trees infecting all cultivated *Prunus* crops. Herbaceous host plants (particularly *Nicotiana* spp.) are suitable for PPV maintenance in laboratory conditions for the virus research (Candresse and Cambra, 2006). PPV is a potyvirus with (+) ssRNA genome coding for a polyprotein, from which at least ten viral proteins are released by proteolytic processing: P1, HC-pro, P3, 6K1, CI, 6K2, VPg, NIa-pro, NIb, and CP. An ORF shift in the P3 region also probably leads to the production of an alternative shorter product P3N-PIPO as documented for other potyviruses (Wei *et al.*, 2010).

Seven strains of PPV are currently recognized from which PPV-M, PPV-D and PPV-Rec have the most substantial impact in Europe. In addition to genome sequence differences, these strains are characterized by specific biological properties including preference of natural host species and different behaviour in herbaceous host plants (Šubr and Glasa, 2008). Infectious cDNA clones of RNA viruses provide an excellent tool for the research of viral gene functions and virus-host interactions (Nagyová and Šubr, 2007). In this work we constructed PPV inter-strain chimeras and applied site-directed mutagenesis for the precise mapping of pathogenicity determinants in the PPV genome.

## Materials and Methods

The PPV cDNA construct pIC-PPV (López-Moya and García, 2000) obtained from Prof. J.A. García (CSIC Madrid) was multiplied in *Escherichia coli* JM109 and purified by the GeneJET Plasmid Miniprep Kit (Fermentas). The QuickChange2 XL Kit (Stratagene) was applied for site-directed mutagenesis according to the manufacturer's instructions. Obtained clones were sequence-verified (Bitcet). *In silico* sequence comparisons (multiple alignments) were performed by the MEGA5 software (Tamura *et al.*, 2007). *N. benthamiana* plants were infected biolistically by the cDNA constructs using tungsten M-10 microparticles (Biorad) and the air-gun method as described (Predajňa *et al.*, 2010). *N. occidentalis* and *N. physaloides* were subsequently infected by mechanical reinoculation from virus-positive *N. benthamiana* the plants were grown at 22°C and 14 h/10 h light/dark period under insect-free conditions. The PPV symptoms were observed 7 – 15 days post infection and the virus presence was proved by immunoblotting with polyclonal anti-PPV antiserum (Šubr and Matisová, 1999).

## Results and Discussion

PPV isolates SK-68 (PPV-M) and BOR-3 (PPV-Rec) (Glasa *et al.*, 2004; Palkovics *et al.*, 1993) have been shown to differ from pIC-PPV (PPV-D, derived from the isolate Rankovic) in type and intensity of symptoms induced in some herbaceous host species (Nagyová *et al.*, 2012). While pIC-PPV caused symptomless infection of *N. physaloides* and mild systemic mottling in *N. occidentalis*, the other two PPV isolates induced local necrotic lesions (LNL) and LNL with systemic vein necrosis, respectively, in these hosts. A set of inter-strain chimeras constructed by exchanging particular fragments in pIC-PPV by corresponding genome parts of BOR-3 and SK68 isolates revealed that different pathotypes of PPV-D (mild) and PPV-M/PPV-Rec (severe) were distinctly connected with the C-terminal part of P1 and N-terminal part of HC-pro (Nagyová *et al.*, 2012).

Multiple alignment of this region from the sequences of pIC-PPV, BOR-3 and SK68 showed that amino acid differences were located exclusively in the C-terminal part of P1, nucleotide variability of the relevant HC-pro gene part was silent (Fig. 1). Four positions were detected in P1 where amino acids differed for mild (pIC-PPV) and severe symptoms-inducing isolates (BOR-3 and SK68). These involved R/W<sup>138</sup>, N/D/H<sup>200</sup>, V/I<sup>254</sup>, and I/V<sup>306</sup>. We presumed that the Val-Ile variability did not influence the protein function because of high similarity and mutual interchangeability of these amino acids. Therefore we focused on the two other positions. Site-directed mutagenesis of pIC-PPV was applied to change the amino acid N<sup>200</sup> to D or H, and R<sup>138</sup> to W in the P1 sequence. All mutants were infectious after particle bombardment of *Nicotiana benthamiana*. Reinoculation on the pathotype test plant species showed that the exchange in position 200 had no influence on the symptom expression. On the other hand, the amino acid exchange in position 138 led to the switch from mild to severe pathotype in both *N. occidentalis* and *N. physaloides*.

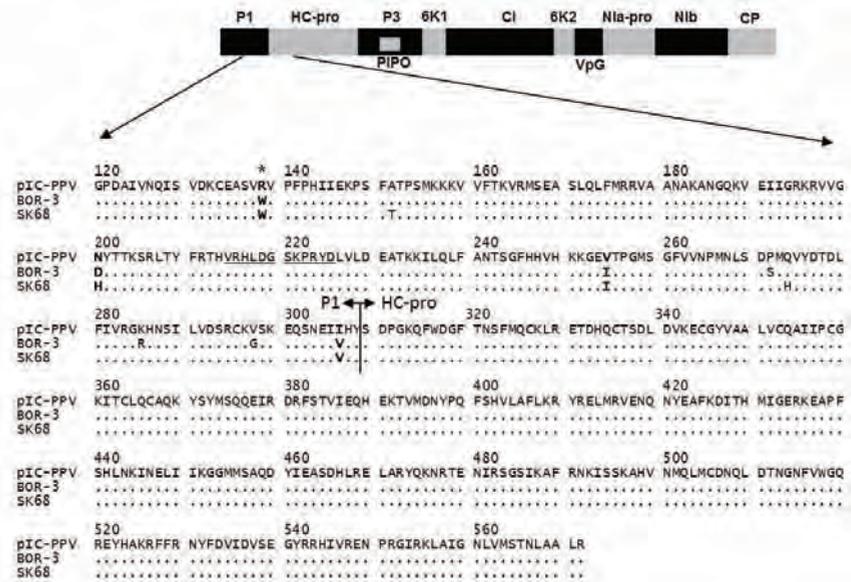


Fig. 1 - Scheme of the PPV genome and alignment of the amino acid sequences derived from the P1/HC-pro genome fragment of three PPV isolates. Identical residues in the alignment are represented by dots. Amino acid polymorphisms correlating with symptomatology are highlighted in bold, the (R/W)138 dimorphism is marked by an asterisk. The border between P1 and HC-pro is indicated by a doublehead arrow.

Very recently, the determinants for similar symptom variance in PPV-PS subisolates (strain PPV-M) were mapped in P1 positions R/W<sup>29</sup> and E/V<sup>139</sup> (Maliogka *et al.*, 2012). Interestingly, our results (R/W<sup>138</sup>) resemble the first of previously published dimorphisms by amino acid residues and the second by the position. This demonstrates that identical differences of the pathotype may be caused by different genome dimorphisms.

Among potyviral proteins P1 is the most variable. Several functions in the virus infection process have been assigned to P1 (Rohořková and Navrátil, 2011). It releases itself from the polyprotein by the serine protease activity of its C-terminal domain (Verchot *et al.*, 1991). The R/W<sup>138</sup> dimorphism lay upstream the protease motifs (Valli *et al.*, 2007), thus the symptom change is probably not connected with protease action. Replacement of P1 in the potyviral genome was able to change the virus host specificity (Salvador *et al.*, 2008). P1 is able to bind RNA (Merits *et al.*, 1998) and it was shown to cooperate with HC-pro in inhibition of the plant defense by RNA silencing (Valli *et al.*, 2006). It is notable that similar mild to severe symptom shift in *N. occidentalis* as recorded in our experiments has also been mapped in the PPV HC-pro (Sáenz *et al.*, 2001). We presume the PTGS inhibition could be modified by point mutations of either P1 or HC-pro leading to visible changes of macroscopic symptoms. As details about the operation of P1 on the cellular level are widely unknown, further research is needed to enable for a better insight on the potyvirus pathogenesis.

### Acknowledgments

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## OCCURRENCE OF *PLUM POX VIRUS* STRAINS IN MORAVIA (CZECH REPUBLIC)

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*Plum pox virus* the causal agent of sharka disease of plums, apricots, and peaches is known to be present in all parts of the Czech Republic. Previous studies have confirmed the presence of three basic strains - *Plum pox virus* strain M (PPV-M), D (PPV-D), and Rec (PPV-Rec) in various *Prunus* species all over Moravia. To obtain detailed information about the distribution of these strains, the survey was done during the period 2005-2011 in this area. In agreement with the expected results mass occurrence of PPV-M was confirmed in most of the intensive peach orchards, its presence was noted in two old apricot orchards and sporadically in myrobalans. In contrary to the surprising data were obtained in the case of PPV-Rec strain. The PPV-Rec was located in two intensive plum orchards originally established from the infected material coming from Serbia (former Yugoslavia). The Spread of PPV-Rec isolates from this primary source proceeded in two different ways. The first was the natural transmission mediated by aphids from infected trees mainly to domestic plums. The second was connected with man as the main vector and the traditional use of offsets (unlucky PPV-Rec infected) for multiplication and plum growing in private gardens and small orchards. Thanks to these two ways of spreading PPV, recently the PPV-Rec has been occurring in narrow zones close to the border with Slovakia along about 80km. To the best of our knowledge PPV-D strain is the most common strain in the whole Moravian region and apart from the above mentioned localities is the dominant strain infecting *Prunus* species.

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## **SURVEY OF SHARKA DISEASE (*PLUM POX VIRUS*) ON STONE FRUIT TREES IN NORTHERN HUNGARY**

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A survey of sharka disease, caused by *Plum pox virus*, was conducted in the Slovakian- Hungarian border region during in 2011. The species surveyed were peaches, apricots, plums on large and small scales farms and blackthorn in its natural surroundings. The virus symptoms were visually assessed except for blackthorn, which was asymptomatic, and leaf samples were taken. Obtained samples were subjected to biological assays with indicator plants, serological (ELISA) and molecular (conventional RT-PCR) diagnostics of PPV. The results indicated that PPV is present in the border region in all the examined stone fruits. The samples were taken from 31 different places within the border region and the infection rate varied between 0 – 100 %. The apricot orchards in the region are less infected by the virus than the plum orchards. The virus was detected in peaches, apricots and plums. The positive isolates were compartmentalized. The definition of the groups is significant, because the isolates from different groups infected the host plants, and spread in the orchards in different measures. We used three processes to define the groups. All tested PPV isolates belonged to the intermediate group according to the symptoms on *Chenopodium foetidum*. Half of the PPV isolates belonged to the recombinant group, 45 % belonged to D and 5 % to M groups according to the molecular characterization and sequence analysis. We obtained got the same results with restriction analysis by *EcoRI* and *DdeI* restriction enzymes.

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## DIVERSITY OF *PLUM POX VIRUS* IN PLUM ORCHARDS IN SERBIA

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Serbia is a leading plum producing country in Europe with more than 41 million of bearing trees. For almost 80 years, *Plum pox virus* (PPV) has been a threat for the stone fruit production in Serbia. So far, three major out of seven strains have been reported: PPV-M, PPV-D and PPV-Rec. Material for this study was collected during three year period (2008-2010). A total number of 265 samples were collected from 84 plum orchards in 12 Serbian districts. Two to four trees showing clear sharka symptoms were randomly chosen and sampled from each orchard. Strain-typing was performed by IC-RT-PCR method with PPV-M and PPV-D specific primers targeting two genomic regions of PPV. Additionally, 39 isolates from different locations were selected for sequencing fragments located in C-ter NIB—N-ter CP part of CP region and C-ter P3-6K1-N-ter CI region. The most prevalent strain in analyzed samples was PPV-Rec (53.5%), followed by PPV-D (27.9%) and PPV-M (5.4%). Mixed infections were found in 13.2% of samples. All types of mixed infections were detected: PPV-M+PPV-D, PPV-M+PPV-Rec and PPV-D+PPV-Rec. The highest incidence among mixed infections was PPV-D+PPV-Rec combination - 67.6%. For the first time, natural triple infection was confirmed in one sample (PPV-M+PPV-D+PPV-Rec). In 37 orchards only one PPV strain was found; in 42 orchards two strains and all three strains were detected in 5 orchards. The most prevalent strain was PPV-Rec which was found in 69 orchards (in single and mixed infections) in all districts. In contrast, the PPV-M strain was found only in 15 orchards. Obtained results confirmed earlier assumptions on the long term presence of PPV-Rec strain on plum in Serbia. Phylogenetic analysis of selected isolates confirmed strain-typing results. Further analysis showed the absence of geographical genetic differentiation of isolates which suggests an intensive gene flow between districts through contaminated planting material in the past.

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## **PLUM POX VIRUS ON SOUR CHERRY IN CROATIA**

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*Plum pox virus* (PPV) has been routinely screened for in Croatian nurseries since 1988 by ELISA. Since 2004, IC-RT-PCR has been introduced to supplement detection methods in accordance with the EPPO protocol. So far, PPV strains M, D, and Rec have been detected in the country with the PPV-M as the geographically most widespread and the most prevalent isolate, especially in the European plum. Sour cherry cultivation has been of increasing importance in the last decade. Besides commercially available sour cherry varieties grown in the continental part of the country, the tendency is to promote the growth of the indigenous variety *Prunus cerasus* L. 'Maraska' in Dalmatia including the preservation of the Maraska gene pool and domestic planting material production. In one of the nurseries specialized for Maraska sour cherry located near Zadar, a routine PPV screening was performed in 2010. Leaf samples were taken from 30 asymptomatic plants and two were found PPV infected by ELISA and IC-RT-PCR. The amplification product obtained by using PPV-C specific primers SoC1/SoC2 resulted in amplification product of expected size (193 bp). The amplicon was cloned into pTZ57R/T vector and inserts from ten recombinant plasmids were sequenced. The genetic structure of this PPV-SoC isolate was homogeneous with all ten amplicon sequences sharing 100% identity. Phylogenetic analysis of 177 nucleotide-long fragments, obtained after removing the primer sequences from amplicons, showed that PPV-SoC isolate from Maraska clusters with other SoC isolates. Interestingly, it also shares 100% nucleotide identity with the prototype sour cherry isolate (GenBank Acc. No. AY184478) originating from Moldova. This is the first report of PPV-C strain in Croatia as well as the first report of PPV in sour cherry.

## A LARGE SCALE SURVEY ON *PLUM POX VIRUS* OF CULTIVATED AND WILD CHERRY IN ROMANIA

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### Abstract

*Plum pox virus* (PPV) is the causal agent of the Sharka disease, one of the most destructive diseases of stone fruits in the world. Seven strains of PPV (D, M, EA, C, W, Rec and T) have been reported so far. Intensive research in the last few years has revealed that D, M and Rec are the most common strains. The other four strains represent additional minor geographically limited PPV groups. PPV-Cherry (PPV-C) is the only strain known to naturally infect sweet and sour cherry. Although PPV-C has been only occasionally detected in a few European countries (Italy, Republic of Moldova, and Romania), the potential introduction into cherry areas currently free of this strain could increase its spread and impact. In Romania, PPV-C was reported in a sweet cherry orchard from the Bistrita area which was promptly destroyed. In order to determine if the PPV-C reported in 2002 was of limited distribution or if it persists in adjacent plots a large survey (349 samples) was conducted that focused on the cherry orchards surrounding the initial plot, where PPV-C was previously found. The survey was extended to other cherry growing areas from six counties of Romania: 190 samples were collected from Iasi and 21 samples each from Sibiu, Valcea, Olt and Constanta counties. Leaves showing diffuse or chlorotic ring spots and also asymptomatic leaves were collected from sweet, sour and wild cherry. A total of 560 samples were tested. Serological tests were conducted using AGDIA ELISA system SRA 31505 and molecular detection was mainly performed by IC-RT-PCR using hPPV(3')NTR/cPPV(3')NTR primer pair. All symptomatic and a few symptomless samples of cherries were also subjected to IC-RT-PCR by using P1/P2 primer pair. All samples tested negative including even some samples showing characteristic PPV symptoms. This survey revealed that the presence of PPV-C in Bistrita area that occurred ten years ago was of limited distribution and was eradicated with the destruction of trees from the original plot. This also supports the fact that PPV-C continues to be of very limited occurrence in cherry trees.

## Introduction

Sharka, caused by *Plum pox virus* (PPV) is considered one of the most destructive diseases of stone fruits in the world. Seven strains of PPV (D, M, EA, C, W, Rec and T) have been reported. PPV-D was originally isolated on apricot while PPV-M was originally characterized on peach (Kerlan and Dunez, 1979). PPV-Rec resulted from recombination between PPV-D and PPV-M (Glasa *et al.*, 2002). Intensive research in the last few years has revealed that D, M and Rec are the most common PPV strains in Europe. The other four PPV strains are geographically or host limited. El Amar (PPV-EA) was originally isolated in Egypt (Wetzel *et al.*, 1991a); Cherry (PPV-C) was isolated from sour and sweet cherry (Kalashyan *et al.*, 1994, Crescenzi *et al.*, 1995, Maxim *et al.*, 2002a); Winnona (PPV-W) was originally isolated in Canada (James and Varga, 2004) but is now known to originate from Eastern Europe (Glasa *et al.*, 2011; Mavrodieva *et al.*, 2012) and PPV-T was isolated from apricot in Turkey (Serce *et al.*, 2009). Additionally, two putative PPV strains were recently described: PPV-CR (Cherry Russian) is the name proposed for a group of unusual isolates able to infect cherry (Glasa *et al.*, 2012), and PPV-An (Ancestor) is the name proposed for an atypical Albanian PPV isolate which is speculated to be the progenitor of PPV-M (Palmisano *et al.*, 2012).

PPV-Cherry (PPV-C) is the strain known to naturally infect sweet and sour cherry (Nemchinov *et al.*, 1996) and to date no other PPV strain has been reported to naturally infect cherry (Wang *et al.*, 2006). PPV-C has been occasionally detected in a few European countries, like Republic of Moldova (Kalashyan *et al.*, 1994) and Italy (Crescenzi *et al.*, 1997) and was also reported in a sweet cherry orchard from Bistrita region of Romania (Maxim *et al.*, 2002a, 2002b) however the cherry plot containing infected trees was destroyed. In an attempt to identify additional trees containing PPV-C that escaped detection in 2002 in the Bistrita region, and in other areas where cherry is grown in Romania, a large scale survey was conducted and the results are presented here for wild and cultivated cherry.

## Materials and Methods

**Orchards surveyed and sampling:** During the vegetative periods (May and June) of 2010 and 2011, the major cherry growing areas in Romania were surveyed and samples of sweet, sour and wild cherry leaves, potentially infected with PPV, were collected. The survey was focused mainly on the Bistrita region in cherry orchards surrounding the initial sweet cherry plot where PPV-Was previously detected (Maxim *et al.*, 2002a). To determine the occurrence of PPV in cherry cultivars in Romania the survey was extended to other cherry growing areas in Iasi, Olt, Valcea, Sibiu and Constanta counties. Trees of sweet, sour and wild cherry were visually monitored and sampled. Symptomatic trees (leaves showing diffuse or chlorotic ring spots) were sampled as a priority. Leaves from these trees were collected at random throughout the plant canopy. In trees with symptoms limited to particular branches leaves were sampled from the symptomatic branches only. The number of symptomatic trees

found was limited so symptomless trees were also sampled. Leaves from symptomless trees were also randomly collected throughout the canopy.

**Serological and molecular diagnoses:** Serological diagnoses were performed by Double Antibody Sandwich-Enzyme Linked Immunosorbent Assay (DAS-ELISA) (Clark and Adams, 1977), using a commercial polyclonal PPV antibody kit according to the manufacturer's instructions (AGDIA, Elkhart, IN). Each sample was tested in duplicate wells. Absorbance values were measured at 405 nm after 1 h substrate hydrolysis. Samples were considered positive if their absorbance values were more than twice those of the negative control. Molecular detection was performed by Immunocapture-Reverse Transcription-Polymerase Chain Reaction (IC-RT-PCR) using pair of primers hPPV(3')NTR / cPPV(3')NTR designed to amplify a fragment from the conservative 3'NCR (NTR) of the PPV genome (Levy *et al.*, 1994). All symptomatic and a few symptomless samples were also subjected to IC-RT-PCR by using P1/P2 primer pair that amplifies a fragment corresponding to the 3'-terminus of the PPV coat protein (CP) gene (Wetzel *et al.*, 1991b). PPV-Was trapped by immunocapture with PPV polyclonal antibodies (Bioreba, Reinach, Switzerland) adsorbed onto a PCR plate (Bio-Rad, Hercules, CA). RT-PCR was conducted using the QIAGEN OneStep RT-PCR Kit (Qiagen Inc., Hilden, Germany) according to the manufacturer's instructions. The thermal cycling scheme used was the following: Reverse Transcription for 30 min at 50°C, denaturation for 2 min at 94°C followed by 35 PCR cycles of template denaturation for 30 s at 94°C, primer annealing for 45 s at 61°C and DNA elongation for 60 s at 72°C. Following the last cycle, amplified DNA was extended for 10 min at 72°C. Amplified products (aliquots of 10µl) were fractionated onto 1.5 % agarose gel electrophoresis in Tris-borate EDTA (TBE) buffer. Bands were visualized by ethidium-bromide staining under UV light.

## Results and Discussion

A total number of 560 samples were collected from 13 plots located in six counties in Romania (Fig. 1). Of the total cherry samples, 460 samples were sweet cherry, 96 were sour cherry, and 4 were wild cherry (Tab. 1).

Prior to sampling a visual survey was conducted and a few trees with characteristic symptoms were observed that suggested potential infection by PPV. However, the serological data did not confirm the presence of the virus both in symptomatic or symptomless samples. The ELISA test showed absorbance values for samples ranging between 0.104-0.264 (OD=405 nm). The absorbance values for negative controls ranged between 0.132-0.235 and positive controls ranged between 1.422 and 3.877. Molecular detection by IC-RT-PCR using both, the hPPV(3')NTR / cPPV(3')NTR primer pair and the P1/P2 primer pair, confirmed the serological results. Except for the positive controls, no PCR products were visualized.

The possibility that the PPV-C detected in a few cherry trees in 2002 in Bistrita could result in the spread of the PPV-C strain into this major cherry growing area could have had a great impact on the production. Additionally, the spread of PPV-C to the wild cherry which could potentially harbor the virus adjacent to the cherry

orchards was also of great concern. PPV is known to have a latent period from the initial infection until the virus reaches detectable levels in trees. The surveys conducted in this study were 10 years after the initial detection of PPV-C in the Bistrita region of Romania. The absence of PPV in the cherry trees tested in this survey using both serological and molecular detection revealed that the initial PPV infection did not spread in the surrounding cherry orchards and also demonstrated the extremely limited potential for PPV-C to occur in other cherry areas of Romania as a result of the find in 2002. The results may also indicate that the presence of PPV-C in the Bistrita area ten years ago was of limited introduction and that the measures taken to destroy the infected cherry plot seems to have been effective in limiting the spread of PPV. PPV-C has been only detected in a limited number of European countries and when detected it is usually very limited. The survey reported here extended surveillance activities into important cherry growing areas in Romania and demonstrated that the PPV-C previously reported (Maxim *et. al.*, 2002a, 2002b) occurred sporadically in a very limited area, did not spread beyond the plot destroyed, and presently does not persist in the surrounding cherry orchards.



Fig. 1 - Counties of Romania from where samples of cherry leaves were collected

Tab. 1 - The samples collected during 2010-2011 from different counties of Romania

Plot no.	Origin (County)	Code (between)	No of samples			
			Total	Sweet cherry	Sour cherry	Wild cherry
A	Bistrita-Nasaud	1-51	51	50	0	1
B	Bistrita-Nasaud	52-130	79	77	1	1
C	Iasi	131-230	100	94	5	1
D	Iasi	231-298	68	68	0	0
E	Bistrita-Nasaud	299-316	18	18	0	0
F	Bistrita-Nasaud	317-418	102	99	2	1
G	Bistrita-Nasaud	419-508	90	2	88	0
H	Iasi	509-530	22	22	0	0
I	Olt	531-538	8	8	0	0
J	Valcea	539-540	2	2	0	0
K	Sibiu	541-545	5	5	0	0
L	Bistrita-Nasaud	546-554	9	9	0	0
M	Constanta	555-560	6	6	0	0
<b>Total</b>			<b>560</b>	<b>460</b>	<b>96</b>	<b>4</b>

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## CHARACTERIZATION OF *PLUM POX VIRUS* ISOLATES FROM DIFFERENT PEACH VARIETIES IN MONTENEGRO

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Although *Plum pox virus* (PPV) is considered the most devastating viral pathogen in stone fruits in Montenegro, data about the occurrence of its strains in the most important peach-growing area is scarce. In September and October 2011, chlorotic ringspots, veinal chlorosis, netting, blotches and deformations, were observed on peach trees in the vicinity of Podgorica. In this study, 58 symptomatic leaves samples originating from 8 different varieties (Rita Star, Morsiani, Caldesi, Adriana, Gloria, Spring Belle, May Crest, Spring Crest, Maria Marta) were collected. The presence of PPV was ascertained in 35 samples (60.3%) using Real-Time PCR. The highest PPV infection rate was detected in Maria Marta and Spring Belle (83.3%), followed by Spring Crest (75%), Adriana and May Crest (66.7%), Gloria and Morsiani (62.5%) and Rita Star (55.5%). PPV was not detected in the Kaldesi variety. In order to determine molecular variability of PPV isolates, four highly infected samples were selected from four varieties (Maria Marta, Gloria, Morsiani and Rita Star). The RT-PCR method with primers targeting CP region, was followed by cloning and sequencing of 1276 bp DNA fragment. Sequence analyses revealed that all peach isolates from Montenegro shared 92.2 – 99.1% nucleotide identity with corresponding PPV-M strain sequences deposited in GenBank. The Montenegrin isolates 374/11 from Rita Star and 399/11 from Gloria were most closely related (99.1 and 97.1%) with isolate SK68 (M92280.1), while isolates 377/11 from Morsiani and 424/11 from Maria Marta shared most identity (97.6 and 98.1%) with previously reported Montenegrin isolate Godinje 1 (HQ452396) from the region of Bar. Considering the high percentage of infected trees in the most important peach-growing area, a severe control related to the importation and production of virus-free propagation material, as well as an eradication of infected trees is of great importance in Montenegro.

## **NEXT-GENERATION SEQUENCING: HISTORICAL PERSPECTIVE AND CURRENT APPLICATIONS IN PLANT VIROLOGY**

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### **Abstract**

Next-generation sequencing technologies, also known as deep or high throughput sequencing, provide a very efficient and fast as well as low cost DNA sequencing beyond the reach of the standard and traditional DNA sequencing technologies developed in the late 1970's. Historical events in the last few centuries that led to its development at the beginning of the 21 century are presented. Next-generation sequencing technologies have been recently applied to the field of plant virology. Application examples are presented, particularly in the areas of virus genome sequencing, ecology, discovery, epidemiology, transcriptomics, replication, detection and identification. Possible prospective for next-generation sequencing in revealing the etiology of the disease of unknown etiology in temperate fruit crops are also discussed. Here we describe the milestones which have marked the evolution of the scientific studies in plant virology starting from the introduction of tobacco plants in Europe

### **Introduction**

Next-generation (high throughput, deep) sequencing technologies have been developed in recent years, especially in the 21st century. These technologies are intended to lower the cost of DNA sequencing beyond those of standard dye-terminator methods. Next-generation sequencing describes platforms that produce large amounts (typically millions to billions) of short DNA reads of a length between 25 and 400 bp. These reads are shorter than the traditional Sanger sequence reads (750 bp). The roots of development of next-generation sequencing go back centuries with the discovery of the New World, the introduction of tobacco to Europe, the development of the science of virology and then molecular virology, revealing the structure of DNA and development sequencing technologies for RNA and DNA.

Here we describe the milestones that have marked the evolution of the scientific studies in plant virology starting from the introduction of tobacco plants in Europe.

**Introductiuon of tobacco plants in Europe:** When Christopher Columbus (1451-1506) discovered the ‘New World’ of the Americas in 1492 he found that Native Americans smoked leaves of a plant species originated in the Americas, tobacco, for medicinal purpose. It was for this reason that the Spanish introduced tobacco to Europe in about 1518. At that time it was claimed that tobacco could cure 36 health problems and in addition to the medicinal benefits of tobacco, the health-giving properties of pipe smoking were emphasized.

**A tobacco disease in Europe that led to the beginning of virology:** Adolf Mayer (1843-1942), a German Chemist was asked by Dutch farmes, when he was the Director of the Agricultural Experiment Station in Wageningen, to study a particular disease affecting tobacco plants. He published a paper in 1886 on the disease which he named ‘mosaic disease of tobacco ‘ and described its symptoms in detail. He demonstrated that the disease cn be transmitted by using the sap from the affected tobacco plants as the inoculum to infect healthy plants. Six years later, in 1892, Dmtri Ivanovski (1864-1920), a Russian botanist, was the first to show that extracts from mosaic diseased tobacco plants could transmit disease to other plants after passing through ceramic filters that retain bacteria. Subsequently, six years later, in 1898, Martinus Beijerinick a Dutch microbiologist and botanist, confirmed and extended Ivanovski’s results. He concluded that tobacco mosaic disease is caused by an infectious agent smaller than a bacterium, which replicates in living plants. He named it a virus and referred to it as *contagium virum fluidum* (soluble living germ or contagious living fluid). This is generally recognized as the beginning of virology

**Tobacco mosaic virus as a model system:** Wendell Stanley an American biochemist, was the first to purify and crystallize a virus that infects either plants (*Tobacco mosaic virus*, TMV) or humans (Influenza virus). He purified, crystallized and described the molecular structure of TMV in 1935, showing that it has properties of both living and non-living matter. TMV was the first virus to be so thoroughly analyzed, beginning a generation of scientific work describing the molecular structures and propagation of other viruses. Stanley’s achievements on TMV were soon reproduced in England by Frederick Bawden, (1908-1972), a plant pathologist and Norman Pirie, a biochemist, who suggested that the presence of 0.5% phosphorus and 2.5% carbohydrate in TMV crystals was not a contaminant but indicated the presence of ‘nucleic acid of the ribose type’ Heinz Fraenkel-Conrat, an American biochemist, from the early 1950’s to 1956, was the first to demonstrate that the replication of a virus (TMV) is controlled by genetic information within its ribonucleic acid (RNA) core. He proved conclusively that TMV RNA was the infectious agent and the protein coat had no infective properties. He was also the first scientist to show that virus molecules, still retaining ‘viral life’, could be thus reconstituted from their separated proteins and RNA In his subsequent studies he showed that once TMV RNA was inside the cell, it took over the host cell’s own genetic material (DNA) and began replicating itself, making not only more infective RNA but compatible protein coatings as well. This study provided definitive proof that RNA can act like DNA as the genetic blueprint for cell replication. In 1957 he published the famous mixed reconstitution experiment, taken as the final

proof that nucleic acids encode protein structure, and not the other way round. In 1960 he determined the complete amino acid sequence of TMV protein--consisting of 158 amino acid residues making it the largest protein of a known structure at the time. His accomplishments were recognized with numerous awards and honors, including the Albert Lasker Award, the first California Scientist of the Year Award in 1958, and many others including The Pope's Pontifical Medal.

### **Major landmarks in nucleic acid sequencing RNA sequencing**

In 1964 Robert Holley (1922-1993), an American biochemist, was the first to sequence an RNA. He determined the sequence and the structure of the 77 ribonucleotides of alanine tRNA, the molecule that incorporates the amino acid alanine into protein. He was awarded the Nobel Prize in Physiology and Medicine in 1968 for this discovery and shared the Prize with Har Gobind Khorana and Marshall W. Nirenberg 'for their interpretation of the genetic code and its function in protein synthesis'. Using the Holley team's method, other scientists determined the structures of the remaining tRNAs. A few years later the method was modified to help track the sequence of nucleotides in various bacterial, plant, and human viruses.

In 1976 Walter Fiers (1931-present), a Belgian molecular biologist, was the first to establish the complete sequence of the 3,569 nucleotides of bacteriophage MS2 RNA.

**DNA sequencing** :In 1953 James Watson, an American geneticist, and Francis Crick, a British physicist discovered the structure of the DNA double helix. The Nobel Prize in Physiology or Medicine 1962 was awarded jointly to James Watson, Francis Crick and Maurice Wilkins 'for their discoveries concerning the molecular structure of nucleic acids and its significance for information transfer in living material'. In 1972, Paul Berg (born 1926), an American biochemist, developed DNA technology, which permits the isolation of defined fragments of DNA. Prior to this, the only accessible samples for sequencing were from bacteriophage or virus DNA. Also, his discovery led to the development of modern genetic engineering. In 1977 Frederick Sanger (born 1918), a British biochemist, was the first to sequence the complete DNA genome of bacteriophage QX 174. He also developed 'DNA sequencing with chain-terminating inhibitor'. Also in 1977 Walter Gilbert (1932-present), an American biochemist and physicist developed 'DNA sequencing by chemical degradation' Paul Berg, Frederick Sanger and Walter Gilbert were the recipients of the Nobel Prize in Chemistry in 1980. The award recognized their contributions to basic research involving nucleic acids. Paul Berg received half of the cash award and the other half was split between Frederick Sanger and Walter Gilbert.

Other major landmarks in DNA sequencing include:

- 1986 LeRoy Hood (USA) announced the invention of the first semi-automated DNA sequencing machine
- 1984 Medical Research Council (MRC) scientists (UK) completed the DNA sequence of the *Epstein-Barr virus* (EBV) 170 kb. EBV causes infectious mononucleosis
- 1987 Applied Biosystems (USA) marketed the first automated sequencing machine, the model ABI 370
- 1990 The International Project on Human Genome was formally started and it was expected to take 15 years. It involved mainly the United States, the United Kingdom, France, Germany, Japan, China, and India.
- 1990 The US National Institutes of Health (NIH) began large-scale sequencing trials on *Mycoplasma capricolum*, *Escherichia coli*, *Caenorhabditis elegans*, and *Saccharomyces cerevisiae*
- 1995 Craig Venter, Hamilton Smith and colleagues (USA) completed the first complete genome of a free-living organism, the bacterium *Haemophilus influenza*. The circular chromosome contains 1,830,137 bases.
- 1996 Pal Nyren and Mostafa Ronaghi (Sweden) develop their method of pyrosequencing
- 1998 Phil Green and Brent Ewing (USA) developed the base calling program 'Phred' for sequencer data analysis
- 2000 Human Genome Project: as a result of widespread international cooperation and advances in the field of genomics (especially in sequence analysis), as well as major advances in computing technology, a 'rough draft' of the genome was finished in 2000 (announced jointly by U.S. President Bill Clinton and the British Prime Minister Tony Blair on June 26, 2000).
- 2001-2003 Human Genome Project: Key findings of the draft (2001) and essentially complete genome were announced in April 2003, 2 years earlier than planned (3.3 billion-base pairs; approximately 23,000 genes).

### **Development of next-generation sequencing platform**

Several companies have been involved in the development of sequencing platforms. In 2000 Massively Parallel Signature Sequencing (MPSS) developed by Lynx Therapeutics (USA) was the first of the next-generation sequencing technologies, thus, *beginning the era of the next-generation sequencing*. The company was later purchased by Illumina

454 Life Sciences (USA) offered a paralleled version of pyrosequencing. The first version of their machine reduced sequencing costs 6-fold compared to automated Sanger sequencing and was the second of a new generation of sequencing technologies, after MPSS. Life Sciences was acquired by Roche.

The 454 GS 20 Roche platform introduced revolutionized DNA sequencing as it could produce 20 million bp (20 Mbp). This was replaced by the GS FLX model in 2007 which is capable of producing over 100 Mbp of sequences in just 4 hours, which increased in 2008 to 400 Mbp

The new model (Roche 454 GS-FLX+ Titanium™ sequencing platform) is now capable of producing over 600 Mbp of sequence data in a single run with Sanger-like read lengths of up to 1,000 base pairs

Illumina (Solexa) developed a sequencing technology based on reversible dye-terminators that uses SBS chemistry. The GAIIX platform generates up to 50 billion bp (50 Bbp) of usable data per run. In 2012, Illumina HiSeq® 2500 had the capacity to sequence a human genome in about 24 hours, ‘Genome in a day’. It can also sequence 20 exomes in a day, or 30 RNA sequencing samples in as little as five hours. It generates 120 billion bp (120 Bbp) of data in 27 hours. Other methods of next-generation sequencing include: Polony sequencing, Ion semiconductor sequencing, DNA nanoball sequencing, Helioscope (TM) single molecule sequencing, Single Molecule SMRT (TM) sequencing: This approach allows read of 1,000 nucleotides

Single molecule real time (RNAP) sequencing, Nanopore DNA sequencing, VisiGen Biotechnologies approach whose expected read length can reach 1,000 nucleotides.

As shown in Fig.1 released by National Human Genome Research Institute, US National Institutes of Health, the cost per raw megabase of DNA sequence and the costs per genome were reduced dramatically and significantly from from July 2001 to October 2011.

In the next few years, it is expected that the third generation of DNA sequencing platforms will increase sequencing capacity and speed while reducing costs. A genome can be sequenced for a very reasonable price (about 1,000 or less US dollars or less) in about 15 minutes. That is about 10% or less than of the present cost.

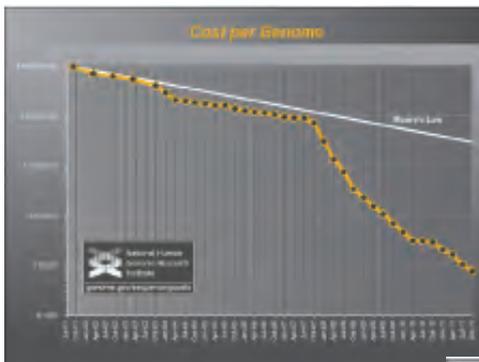
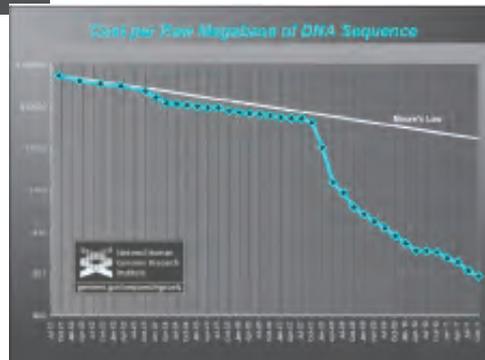


Fig.1 - Cost in US Dollars per raw megabase of DNA sequence (left). Cost per genome from July 2001 to October 2011 (Right).



### Biological applications of next-generation sequencing

To date the biological applications of next-generation sequencing have focused primarily on *de novo* sequencing of genomics, re-sequencing genomics, metagenomics\*, and sequencing the transcripts of an organism (RNA-seq)\*\*

Next-generation sequencing technologies combined with sophisticated bioinformatics have been recently changing the field of plant virology, particularly in the areas of genome sequencing, ecology, discovery, epidemiology, transcriptomics, replication, detection and identification. Currently a limited number of plant viruses and viroids have been identified from infected tissues and sequenced by RNA-seq.

Both total nucleic acid and total double strand RNA (dsRNA) from virus infected tissue were isolated and sequenced; in some cases host nucleic acid was partially eliminated by hybridization to material from healthy plants to enrich for virus sequence from infected plant material prior to sequencing. All the viruses discovered in infected plants or viruliferous vectors using next-generation sequencing are termed 'viromes'. These technologies have enabled viruses infecting several plant species, a viroid infected grapevine and viruses in viruliferous vectors to be analyzed.

Plant viruses or viroids can also be detected indirectly. In response to infection by RNA/DNA viruses or viroids, the host plant generates specific RNA molecules, 21 to 24 nt in length, called short interfering RNAs (siRNA). RNA silencing (RNAi) is a cytoplasmic cell surveillance system to recognize dsRNA and specifically destroy single and double stranded RNA molecules homologous to the inducer, using small interfering RNAs as a guide (see Mlotshwa *et al.*, 2008; Barba and Hadidi, 2009; for recent reviews). Next-generation sequencing of siRNAs offers good opportunities to identify viruses or viroids infecting plants, even at extremely low titers, symptomless infection, and including previously unknown viruses or viroids. Next-generation sequencing can provide thousands to millions of siRNA sequences from virus or viroid infected plant materials. When virus or viroid-derived siRNAs are abundant enough, virus or viroid genome fragments can be assembled. This approach requires relatively sophisticated bioinformatics. Since the virus or viroid siRNAs are 21–24 nt in length, their sequences can be employed directly as primer sequences to amplify viral or viroid fragments by PCR or RT-PCR.

Tables 1-2 summarize the use of next-generation sequencing from 2009 to 2012. The first articles on the use of next-generation sequencing in plant virology were published in 2009.

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\* the term 'metagenomics' was first used in publications in 1998. Subsequently, in 2005 Kevin Chen and Lior Pachter (at University of California, Berkeley) defined metagenomics as 'the application of modern genomics techniques to the study of communities of microbial organism directly in their natural environments, bypassing the need for isolation and lab cultivation of individual species.

\*\* Sequencing of the cDNAs of diverse RNAs (genomic, mRNA, RNAi etc.) has been coined RNA-seq (Wang *et al.*, 2009)

### **Prospects for next-generation sequencing in revealing the etiology of diseases of unknown etiology in temperate fruit crops**

Most, if not all, diseases of unknown etiology that affect stone, pome and small fruit crops are graft transmissible. This implies that systemic pathogens (viruses, viroids, and phytoplasm) are most likely involved in the etiology of these diseases. It is also possible that an un-described class of pathogen may also be involved in the disease etiology. Currently there are over 35 diseases of unknown etiology that affect stone fruits and similar number that affect pome fruits (Howell *et al.*, 2011). Examples of the major diseases of unknown etiology that affect stone fruits are peach wart, cherry stem pitting and plum fruit crinkle, and the ones that affect pome fruits are apple green crinkle, apple rubbery wood and dead spur. As revealed by next generation sequencing, Yoshikawa *et al.*, 2012 reported that apple green crinkle is a disease complex and at least 5 viruses and 1 viroid may be associated with it. Among major diseases of unknown etiology that affect small fruits are blueberry fruit drop, blueberry bronze leaf curl, blackberry yellow vein, raspberry leaf curl, raspberry yellow spot and strawberry latent C. Thekke-Veetil *et al.*, 2012 identified a novel *ampelovirus* as one of the viruses associated with blackberry yellow vein disease as revealed by next-generation sequencing.

### **Conclusions**

Next-generation high throughput sequencing technologies have become available at the onset of the 21 century. They are continually being improved to become faster, more efficient and cheaper. Since 2009 have been used in many fields of biology and utilized in several areas of plant virology. Detection, identification of known and unknown viruses and/or viroids in infected plants, currently are ones of the most successful applications of the next-generation technologies. It is expected that these technologies will play very significant roles in the next few years in many research and non-research areas of plant virology.

Tab. 1 - Next-generation sequencing of isolated siRNA, total RNA, viral RNA or DNA from herbaceous or grass hosts infected with viruses or viroids or from viruliferous vector

Host	Virome	Target used	Sequencing platform	Reference
Sweet potato	Detected: <i>Sweet potato feathery mottle virus</i> , <i>Sweet potato chlorotic stunt virus</i> ; Discovered: two badnaviruses (dsDNA), one mastrevirus (ssDNA)	si RNAs	Illumina	Kreuze <i>et al.</i> , 2009
<i>Gomphrena globosa</i>	Plants were inoculated with an unknown virus. A new <i>Cucumovirus</i> was identified. Proposed name: <i>Gayfeather mild mottle virus</i>	Total RNA + Subtractive hybridization	Roch4 454 GS-FLX	Adams <i>et al.</i> , 2009
Cucumber	To study <i>Hop stunt viroid</i> pathway involved in the biogenesis of the viroid siRNAs	siRNAs	Illumina	Martinez <i>et al.</i> , 2010
<i>Nicotiana benthamiana</i>	RNA-dependent RNA polymerase 6 restricts accumulation and precludes meristem invasion of <i>Potato spindle tuber viroid</i> which replicates in nuclei	Plant and viroid sRNAs	Illumina EAS269 GAI	Di Serio <i>et al.</i> , 2010
Cassava	The complete sequence of the Tanzanian strain of <i>Cassava brown streak virus</i> was determined and compared with that of the Ugandan strain. The virus is highly heterogeneous at both the isolate and strain levels with nucleotide identity at the isolate level of 76%	Total RNA + subtractive hybridization	Roche 454 GS-FLX	Monger <i>et al.</i> , 2010

follow					
Pepper eggplant	The complete nucleotide sequences of two new viruses <i>Pepper yellow leaf curl virus</i> ( <i>Polerovirus</i> ) and <i>Eggplant mild leaf mottle virus</i> ( <i>Ipomovirus</i> ) were determined	Purified virions viral RNA	SOLiD	Dombrosky <i>et al.</i> , 2011	
Wild Cocksfoot Grass	<i>Cereal yellow dwarf virus</i> ( <i>Luteovirus</i> ) discovered infecting this grass (not previously reported infecting this host), <i>Cocksfoot streak virus</i> ( <i>Potyvirus</i> ) was detected	siRNAs	Roche 454	Pallet <i>et al.</i> , 2010	
Tomato	A novel <i>Potyvirus</i> with only 70% identity to known viruses was identified from Mexican samples. Also two strains of <i>Pepino mosaic virus</i> sharing only 82 % identity were detected in US samples	siRNAs	Illumina GAllx	Ling <i>et al.</i> , 2011	
Different hosts	A novel virus infecting watercress was identified (proposed name: <i>Watercress white vein virus</i> ). Viruses such as <i>Piper yellow mottle virus</i> , <i>Arachacha virus B</i> , and <i>Potato black ring virus</i> were detected and sequenced	Partial virus and RNA purification then fragmented	Roche 454 GS-FLX	Mumford <i>et al.</i> , 2011	
Cultivated and wild plants	Four virus species were discovered and were proposed as members of the genera <i>Potyvirus</i> , <i>Sadwavirus</i> , and <i>Trichovirus</i> . Twelve virus species were previously described (genera: <i>Potyvirus</i> , <i>Nepovirus</i> , <i>Allexivirus</i> , and <i>Carlavirus</i> ). Virus sequences were subsequently matched to original host plants using RT-PCR assays	Total RNA from pooled samples. Poly-A RNA was isolated	Illumina	Wylie <i>et al.</i> , 2011	

follow

Tobacco cv Xanthi nc	Identified gene expression changes associated with disease development in tobacco plants induced by infection with the M strain of <i>Cucurbit mosaic virus</i> . Sequencing analysis of tobacco transcriptome identified 95,916 unigenes, 34,408 of which were new transcripts by database searches.	Total RNA then treated with DNase I	Illumina Hi Seq 2000	Lu <i>et al.</i> , 2012
<i>Bemisia tabaci</i>	Four novel <i>Begomoviruses</i> were discovered in their viruliferous vector	Purified viral DNA	Metagenomic reads 100-700 nt	Ng <i>et al.</i> , 2011
Grapevine leafhopper	A novel <i>Meravivirus</i> associated with grapevine syrah decline was detected in the vector	Total nucleic acids of the viruliferous vector	Roche 454	Al Rawahmih <i>et al.</i> , 2009

Tab. 2 - Next-generation sequencing of isolated siRNA, total RNA or dsRNA from grapevine, fruit trees or small fruits infected with viruses or viroids

Host	Viroome	Target used	Sequencing platform	Reference
Grapevine	A novel <i>Maravivirus</i> identified ( <i>Grapevine Syrah 1</i> ) associated with grapevine syrah decline. The virus was also identified in leafhopper vector. Also detected in plant tissue RSPAV, GRVfV, GLRAV 9, AGVd, HSVd, GYSVd <i>Grapevine virus E</i> , not previously in S. Africa. A mycovirus similar to <i>Penicillium chrysogenum virus</i> , two other mycoviruses, GLRAV 3, DRSPAV, GVA Viruses of the genera <i>Foveavirus</i> , <i>Macularivirus</i> , <i>Marafivirus</i> , and <i>Nepovirus</i> were detected. siRNAs originate from both genomic and antigenomic strands with the exception of tymoviruses, the majority are derived from antigenic virus strand Twenty six fungal groups were identified in a single plant source. Three of the mycoviruses were associated with <i>Boutyitis cinerea</i> . Most of the rest were undescribed A novel viroid-like circular RNA of 375 nt was identified that has no significant homology with known molecules and encodes an active hammerhead ribozyme in both plus and minus polarities	Total RNA o dsRNA	Roche 454	Al Rwahmih <i>et al.</i> , 2009
Grapevine		dsRNA	Illumina	Coatsee <i>et al.</i> , 2010
Grapevine		siRNAs	Illumina	Pantaleo <i>et al.</i> , 2012
Grapevine		dsRNA	Roche 454	Al Rwahmih <i>et al.</i> , 2009
Grapevine		siRNAs	Illumina	Ding, 2011

follow

Host	Virome	Target used	Sequencing platform	Reference
Citrus	In <i>Citrus tristeza virus</i> (CTV) infected citrus plants it was shown that the citrus homologues of Dicer-like ribonucleases mediate the genesis of the 21 and 22 nt CTV siRNAs and that the ribonucleases act not only on the genomic RNA but also on the 30 co-terminal subgenomic RNAs and, particularly, on their dsRNA forms. A novel citrus miRNAs was also identified and how CTV influences their accumulation was determined	CTV siRNAs, gRNA sgrNAS	Illumina	Riuz-Ruiz <i>et al.</i> , 2011
Citrus	A novel virus related to <i>Begomovirus</i> identified. It is associated with citrus chlorotic disease originally described in turkey in 1986.	siRNAs	Illumina GAllx	Hartung <i>et al.</i> , 2010
Peach	To study the genesis of <i>Peach latent mosaic viroid</i> siRNA and viroid pathogenesis	siRNAs	Illumina	Di Serio <i>et al.</i> , 2009
Peach and Apple	<i>Apple mosaic virus</i> , <i>Apple stem pitting virus</i> , <i>Peach necrotic ringspot virus</i> , <i>Peach latent mosaic viroid</i> , and <i>Citrus exocortis viroid</i> were positively identified in multiple peach and apple tissues. Large numbers of 20-22 nt reads showed no identity to known viruses or viroids suggesting the presence of additional unknown species. Only a small portion of viruses siRNAs identified . In contrast siRNAs of PLMVd were easily detected	siRNAs	Illumina	Dradik <i>et al.</i> , 2011
<i>Prunus</i>		siRNAs	Illumina SOLID	Olmos <i>et al.</i> , 2012

Host	Virome	Target used	Sequencing platform	Reference
follow				
<i>Prunus</i>				
	PPV, PNRS, etc. and novel virus agents	dsRNA	Roche 454	Candresse <i>et al.</i> , 2012
Apple, Citrus, Grapevine	Detected ASPV, ACLSV and an unknown mycovirus. Detected two variants of CTV and ASGV. Detected variants of GLRaV-3, GVA and an unknown mycoviruses	siRNAs	Illumina	Maree <i>et al.</i> , 2012
Apple	Identified agents associated with green crinkle diseased of apple trees. The disease is a complex one as the following agents were found to be associated with it: ASGV, ASPV, ACLSV, ApLV, ApPCLSV and CEVd ( <i>Citrus exocortis viroid</i> )	siRNAs	Illumina	Yoshikawa <i>et al.</i> , 2012
Fig	Detected <i>Fig mosaic virus</i> and <i>Fig latent virus-1</i> for their elimination from infected clones. This was the first application of next-generation sequencing technology to detect and identify known and new species of viruses infecting fig trees.	dsRNAs	Illumina	Chiumenti <i>et al.</i> , 2012
Blackberry	A novel <i>Ampelovirus</i> in the family <i>Closteroviridae</i> was identified as one of the viruses associated with blackberry yellow vein disease complex.	dsRNAs	Illumina	Thekke-Veetil <i>et al.</i> , 2012

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## **DIRECT METHODS OF SAMPLE PREPARATION FOR DETECTION OF FRUIT TREE VIRUSES AND VIROIDS BY REAL-TIME RT-PCR AMPLIFICATION**

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Biological indexing and serological methods are widely used in fruit tree viruses and viroid detection. Molecular techniques have revolutionized plant pathogen detection and identification and in particular real-time RT-PCR which is widely known as the most sensitive and reliable method. However, conventional real-time RT-PCR requires a RNA purification step that makes this procedure tedious, costly, time-consuming and not suitable for large-scale analyses. Fast and simple sample preparation procedures have been developed and patented for real-time amplification of fruit tree viruses and viroids. Three direct methods of sample preparation prior to molecular analyses were compared and validated using *Plum pox virus* (PPV) and *Peach latent mosaic viroid* (PLMVd) as models. Two of these methods are based on the use of crude plant extracts: i) spotting of crude extracts on a nylon positively charged or Whatman membrane, and ii) dilution of crude extracts in an extraction buffer. The third method avoids the preparation of crude extracts: iii) tissue-print or squash that consists in pressing plant tissues on a piece of membrane. These three sample preparation methods can be used to store immobilized targets, either frozen or at room temperature, for long time periods without decreasing the accuracy of detection. This new methodology was compared with conventional real-time RT-PCR and hybridization for PPV and PLMVd detection, respectively. Statistical parameters such as kappa Cohen index, likelihood ratios and predictive positive and negative values were used as diagnostic parameters for validation purposes. Direct sample preparation methods, combined with real-time RT-PCR proved to be very sensitive, specific and accurate. Two complete commercially available kits for PPV or PLMVd detection by real-time RT-PCR, based on immobilized targets, were successfully evaluated.

## VALIDATING PCR PROTOCOLS FOR THE DETECTION OF STRAWBERRY VIRUSES IN AUSTRALIA

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To assist the integration and implementation of the PCR assays in pathogen testing protocols for the production of certified strawberry runners in Australia, a virus inoculated ‘dummy nucleus’ (‘DN’) of two strawberry varieties was created in December 2008. The ‘DN’ was maintained in a temperate climate in a similar manner to the Victorian nucleus collection and used to validate PCR assays for the detection of *Strawberry mottle sadwavirus* (SMoV), *Strawberry crinkle cytorhabdovirus* (SCV), *Strawberry mild yellow edge potexvirus* (SMYEV), *Strawberry vein banding caulimovirus* (SVBV), *Beet pseudos yellows crinivirus* (BPYV), and *Strawberry pallidosis associated crinivirus* (SPaV). In 2010/11 the ‘DN’ was also replicated in a subtropical climate in Queensland. The ‘DN’ plants were tested once each year by biological indexing and were tested monthly PCR. PCR testing of the 2008/09 Victorian ‘DN’ showed that viruses were not reliably detected in strawberry plants during the first six months post-inoculation. Reliable detection was only achieved during the following seasons and the rate of virus transmission from mother to daughter plants reach 100%. Virus testing during spring (October-December) of the Victorian ‘DN’ plants during 2009/10 and 2010/11 was the most reliable time for virus detection. In some years autumn (March-May) may also be a reliable time for virus detection by PCR. The results for the Queensland ‘DN’ collection during the 2010/11 season indicated that there may not be a trend for the timing of detection of all virus species and no single month or season appears more reliable than another for virus detection. The results from Victoria and Queensland indicated that biological indexing is less reliable for virus detection than PCR techniques as many of the inoculated indicators that were expected to show symptoms were symptomless even though viruses could be detected by PCR. However, the PCR tests were also not 100% reliable because there were only a few months of the year in which viruses were detected in all known infected plants. This highlights the importance of timing for testing and using a combination of molecular and biological tests for certification.

**CHARACTERIZATION OF DIVERGENT *PLUM BARK  
NECROSIS STEM  
PITTING-ASSOCIATED VIRUS* ISOLATES AND  
DEVELOPMENT OF A POLYVALENT PBNSPaV-SPECIFIC  
DETECTION TEST**

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Plum bark necrosis/stem pitting was first reported in California in 1986 on *Prunus salicina* cv. Black Beaut. The putative causal agent was later characterized and identified as *Plum bark necrosis and stem pitting-associated virus* (PBNSPaV), belonging to the *Ampelovirus* genus of the *Closteroviridae* family. This virus is now reported to infect many stone fruit species in European countries but also, more recently in China. To better understand PBNSPaV diversity, we pyrosequenced the complete genomes of divergent PBNSPaV isolates from 3 sources: a Japanese plum (*Prunus salicina*) collected in France but from uncertain origin; a Japanese plum (*P. salicina*) affected by the Peach red marbling disease (PRM) and a peach (*P. persica*) from the Nanjing region in China. The genomic RNAs of these divergent isolates show the same genomic organization as the PBNSPaV reference isolate. The less divergent isolates, found in the Japanese plum and in the PRM source, are closely related to each other but only about 82.8% identical to the PBNSPaV reference isolate. On the other hand, they are highly homologous with two recently described PBNSPaV divergent isolates from China (98.9-100% aa identity in the partial HSP70 region). The most divergent isolate, from the Chinese peach source, shows only about 71.2% identity to other PBNSPaV isolates and is not detected by currently available PBNSPaV detection techniques. The complete sequencing of these divergent isolates identified a conserved genomic region in the P61 gene and the development of a new primer pair with improved polyvalence. Taken together these results indicate a much broader diversity of PBNSPaV than previously thought and provide for a more robust detection of this still poorly characterized pathogen.

## A FIRST EVALUATION OF siRNA NEXT-GENERATION SEQUENCING FOR DETECTION AND CHARACTERIZATION OF PRUNUS VIRUSES

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The plant post-transcriptional gene silencing antiviral defense mechanism results in the generation by plant Dicer-like enzymes and in the accumulation of small, 21-24 nucleotide long RNAs derived from viral genomes. These so called small interfering RNAs (siRNAs) can be sequenced with an extremely high throughput using the Illumina next generation sequencing technology. Analysis of such siRNA populations has been reported to allow the efficient detection of plant viruses in samples of unknown sanitary status. We evaluated this strategy on a few multiple infected *Prunus* samples. While high numbers of siRNAs could be readily sequenced, only a low proportion of siRNAs of viral origin could be identified. On the other hand, high amounts of siRNAs derived from the genome of *Peach latent mosaic viroid* were detected, allowing the reconstruction of the whole genome of the isolate present. Various strategies were used to analyse the virus-derived siRNAs, allowing an effective assessment of the sanitary status of the *Prunus* samples analysed but showing only a partial ability to reconstruct viral genomes without a priori information.

## USE OF 454 PYROSEQUENCING FOR THE FAST AND EFFICIENT CHARACTERIZATION OF KNOWN OR NOVEL VIRAL AGENTS IN *PRUNUS* MATERIALS

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While detection of a well-known virus in *Prunus* material is now relatively straightforward, the task of performing a full indexing for the presence of any known or novel viral agent remains a challenge, due to the wide variability of plant viruses, the large number of viruses infecting *Prunus* crops and the fact that many of them remain uncharacterized. Besides biological indexing, which has a broad polyvalence but is lengthy and labor/cost intensive, there is therefore a need for novel approaches for the detection, without any prior information, of all viruses present in a given *Prunus* host. Novel sequencing technologies, which generate massive amounts of data at a fraction of the cost and effort of previous ones, offer great opportunities for virus identification and characterization. We developed an indexing strategy based on the pyrosequencing of purified double stranded RNAs. The procedure, from dsRNA purification to random amplification prior to sequencing, was streamlined and can be performed in a few days using only basic laboratory equipment. A strategy for the analysis of the large amounts of sequence data produced was also developed. An automated data processing pipeline is being developed, to allow data analysis in laboratories with limited bioinformatics expertise. Thanks to a multiplexing approach, the analysis currently costs 300-400 euros per sample, including salaries, which compares favourably with the full cost of a biological indexing. Using this strategy, we found multiple infections in *Prunus* materials, implicating both well known viruses (PPV, PNRSV) and novel viral agents. The procedure takes a few days and appears robust, although further testing is clearly needed. It could be applied both in the research laboratory for the fast characterization of novel viruses and in plant quarantine, germplasm collections and certification schemes for the validation of the health status of valuable *Prunus* material.

## THE STUDY OF PLANT VIRUS DISEASE ETIOLOGY USING NEXT-GENERATION SEQUENCING TECHNOLOGIES

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The use of next-generation sequencing (NGS) has been established as a reliable approach to study the genetic composition of an array of environmental samples. This technology has been applied in numerous studies ranging from microbe populations in the oceans, to the virus complexity of grapevine diseases. In plant pathology NGS technologies have not been limited to metagenomic sequencing for pathogen discovery, but have also been used for other applications such as the study of plant-pathogen interactions. In this study, we used a metagenomic sequencing approach on dsRNA to examine the virus etiology of a variety of diseased fruit crops with NGS. Samples with known disease profiles were selected for this proof of principle experiment. Double-stranded RNA was extracted from the cambium tissue of virus diseased apple, citrus and grapevine plants with a cellulose extraction protocol and sequenced in an unbiased manner using the Illumina sequencing-by-synthesis technology on a HiScanSQ. The apple sample contained multiple latent viruses and was routinely used as a positive control in ELISA diagnostics, the citrus sample displayed typical *Apple stem grooving virus* symptoms and the grapevine samples, typical leafroll disease symptoms. Approximately 3 million reads for each sample were generated and used in the *de novo* assemblies. Assembled contigs were subjected to BLAST searches against the NCBI non-redundant DNA and protein databases to identify and classify contigs according to the sequence they aligned to with the highest bit score. For each of the samples the virus profile was confirmed as with other techniques. In the apple sample the expected viruses (ASPV and ACLSV) were identified as well as an unknown mycovirus whereas in the citrus sample two genetic variants of CTV were identified as well as the expected ASGV. For the two grapevine samples multiple virus infections from different genetic variants (GLRaV-3, GVA) were identified including unknown mycoviruses. The results of this proof of principle experiment clearly indicate that the metagenomic sequencing approach of dsRNA can be successfully implemented to study plants with unknown virus etiology.

## VIRUS SANITATION AND DEEP SEQUENCE ANALYSIS OF FIG

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Fig (*Ficus carica* L.) is one of the most widespread fruit tree in the Mediterranean basin. Despite the significance of the crop in some areas, its sanitation status has deteriorated as several new viruses affecting this perennial woody host have recently been discovered and characterized. No certification programme for the multiplication of virus-free propagative material has been currently applied in fig-growing countries. In order to create sanitarily-improved commercial orchards using virus-tested primary stocks, the sanitation of some accessions (mainly showing fig mosaic symptoms and verified as being affected by *Fig mosaic virus*, FMV, and *Fig Latent Virus-1*, FLV-1) was applied through sanitation treatments (*in vitro* heat therapy and shoot tip culture, alone or in combination). RT-PCR analysis was carried out before and after treatments. Furthermore, a real time RT-PCR protocol, SybrGreen-based, was developed for the detection of FLV-1. Double stranded RNA, extracted from the accession F5P5 (from which FLV-1 genome was firstly sequenced), was used to construct a cDNA library for deep sequencing (by HiScanSQ Illumina technology) to analyze the whole viral population. Several contigs of 6 different viruses, belonging to different taxa, were identified after alignment to a sequence database and their presence was further validated by molecular tools. This study enabled (i) the production of fig clones in which FMV and FLV-1 were eliminated; (ii) the development of a new sensitive tool for FLV-1 detection to be applied for phytosanitary controls; and (iii) the first application of a whole genomic analysis technique to check virus presence and new species identification in fig.

## DEVELOPMENT OF QUANTITATIVE REAL-TIME RT-PCR FOR THE DETECTION OF *HOP STUNT VIROID*

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*Hop stunt viroid* (HSVd) is a worldwide pathogen able to infect a wide range of hosts. It affects latently grapevine and almond, whereas in hop, plum, peach, apricot, cucumber and citrus it causes specific disorders such as hop stunt, dapple fruit on plum and peach, fruit rugosity on apricot and plum, pale fruits on cucumber and cachexia on citrus. In some cases different molecular variants have been associated with a peculiar symptom expression such as the cachexia isolate on citrus. Because of the diffusion and the wide range of hosts of HSVd, its detection requires the development of a diagnostic protocol able to identify all viroid isolates from every host. For this reason, a new protocol for HSVd detection was developed by designing a new primer pair and a probe in order to perform a real-time RT-PCR assay based on TaqMan™ chemistry. The protocol was tested for its sensitivity and specificity. Sensitivity was evaluated with a dilution series of *in vitro* transcripts from a whole cloned HSVd genome. Dilutions were used for the production of a standard curve able to quantify the viral template in infected samples. The method was also evaluated for its specificity using target controls from plum, peach, citrus (cachexia and not cachexia isolates) and grapevine isolates and non-target controls from healthy peach, apricot, plum, pear, citrus, almond and grapevine and other viroids (*Apple scar skin viroid*, *Peach latent mosaic viroid*, *Pear blister canker viroid* and *Potato spindle tuber viroid*). According to the results obtained to quantify the viral pathogen the protocol revealed a high-quality value of efficiency. These results underline the suitability of the new diagnostic protocol for HSVd detection and quantification.

**APPLICATION OF HIGH RESOLUTION MELT (HRM)  
ANALYSIS FOR SIMULTANEOUS DETECTION OF *CHERRY  
GREEN RING MOTTLE VIRUS* AND *CHERRY NECROTIC  
RUSTY MOTTLE VIRUS***

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*Cherry green ring mottle virus* (CGRMV) and *Cherry necrotic rusty mottle virus* (CNRMV), unassigned members of the family *Flexiviridae* are flexuous, filamentous plant viruses with a single-stranded, positive-sense RNA genome. Both viruses have a very similar morphology and genomic organization and share about 60% identity at the nucleotide level over the entire genome. The purpose of this study was to develop a rapid and sensitive method for simultaneous detection of CNRMV and CGRMV by high resolution melt (HRM) RT-PCR. The CNRMV and CGRMV isolates originated from sweet and sour cherry cultivar collections maintained in the Research Horticulture Institute and from commercial orchards in Poland. Seven sources of CNRMV, eight of CGRMV and two containing mix infection were used in this study. Total nucleic acids were isolated from the leaves using the silica capture method. Real-time PCR and HRM analysis were performed using Light Cycler 480 II system (Roche Diagnostics, Mannheim, Germany). Detection of CNRMV and CGRMV was performed simultaneously using a probe free, multiplex reaction which included one specific primer set for each virus or one primer pair specific for both of them. These two strategies allowed us to detect virus infection in all the tested samples. In addition, HRM analysis made it possible to differentiate clearly between CNRMV and CGRMV by HRM curves. When the HRM profiles were displayed in normalized melting curve format, three distinctly different patterns could be seen corresponding to one for each individual virus and one for the virus mix. Monophasic curves were generated from the samples that were positive for one virus only, while biphasic curves were observed from mixed templates. Moreover, sequence variations among CNRMV and CGRMV isolates were observed from the HRM peaks, as confirmed by sequencing. This first application of HRM RT-PCR in plants reveals a new potential for rapid and sensitive detection of multiple pathogens.

## DETECTION OF *PLUM POX VIRUS* WITH IMMUNOSENSOR UTILIZING ANTIBODIES IMMOBILIZED ON GOLD NANOPARTICLES

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An immunosensor was developed for fast and sensitive detection of *Plum pox virus* (PPV) in plant extracts. It was based on gold electrodes modified with: 1,6-hexanedithiol, gold nanoparticles, anti-PPV polyclonal antibody and bovine serum albumine (BSA). It was used for the determination of the presence of the virus in the extracts from plum (*Prunus domestica*) and tobacco (*Nicotiana benthamiana*) leaves. PPV particles trapped from the solution by antibody immobilized on the electrode formed an insulating layer with resistance dependent on the quantity of virions deposited. This phenomenon was monitored by electrochemical impedance spectroscopy (EIS). As measured in a series of experiments, the immunosensor displayed a very good detection limit about of 10 pg/ml and a wide dynamic range from 10 pg PPV/ml to at least 200 pg/ml. The presence of extracts from plant materials had no influence on the immunosensor response. The immunosensor was capable of discriminating between samples from healthy plants and samples containing 0.01% of extract from infected plant material. These results make the immunosensor a promising device for fast, reliable and simple PPV-Detection. With the use of soecific antibodies for other viruses the potential range of applications in phytodiagnosics seems to be vast, although many details need to be worked out and practical suitability needs to be carefully validated. The work on the miniaturization of the immunosensor presented is in progress.

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## **DETECTION OF TWO STRAINS OF ‘*CANDIDATUS PHYTOPLASMA ASTERIS*’ FROM PEACH AND APRICOT USING LOOP-MEDIATED ISOTHERMAL AMPLIFICATION**

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Phytoplasmas are associated with more than 700 diseases in several hundred plant species and cause significant damage to agricultural crops. ‘*Candidatus Phytoplasma asteris*’, one of the largest group of phytoplasma species, infects many crops, including fruit trees. Since there is no effective way to cure phytoplasma diseases, early detection and the removal of infected plants is important to prevent their spread. PCR is the most commonly used method for detecting phytoplasmas due to its high sensitivity and specificity; however, it is slow and requires expensive equipment, including a thermal cycler and electrophoresis apparatus, which makes it difficult to apply it in the field. In this study, we developed a LAMP-based detection kit for phytoplasmas (‘*Ca. P. asteris*’ and ‘*Ca. P. japonicum*’). The LAMP kit targets the *groEL* gene, a highly conserved bacterial housekeeping gene (> 93.8% nucleotide identity among ‘*Ca. P. asteris*’ strains). Using this kit, we attempted to detect the ‘*Ca. P. asteris*’ PYR and AY-A strains, which were transmitted to periwinkle from peach yellows and apricot chlorotic leafroll infected peach and apricot trees, respectively. First, we purified total DNA from PYR- and AY-A-infected periwinkle leaves and from healthy periwinkle, peach and apricot leaves. Using these DNA samples, the LAMP reaction was performed at 61°C for 60 min. Both phytoplasma strains were specifically detected. Using a real-time LAMP assay, we confirmed that amplified DNA from the PYR- and AY-A-infected samples could be detected in about 30 and 36 min, respectively. A LAMP-based detection kit would be useful for the easy, rapid detection of ‘*Ca. P. asteris*’.

## DEVELOPMENT OF A MINI-OLIGO ARRAY FOR THE ANALYSIS OF *PLUM POX VIRUS* VARIABILITY

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The SharCo project (European Community's Seven Framework Programme (FP7/2007-2013) under Grant Agreement n°204429) involves multidisciplinary approaches to provide strategies and tools for *Plum pox virus* (PPV) containment. In this context, one of the tasks aimed at the development of a mini-oligo array approach for the rapid, genome wide analysis of PPV isolates. A first generation of the oligochip containing 32 oligoprobes was produced and allowed discrimination among PPV strains. The development of a second generation of PPV oligochips (containing 10 replicates of 90 probes from 18 to 22 nucleotides each) then led to the successful analysis and probe of the intra-strain variability. A complete protocol including spotting of validated probes, post-spotting procedures, hybridization, evaluation of signals using a GenPix 4000B scanner and final signal analysis, was generated. A rapid characterization of PPV-Diversity and rapid PPV typing based on mini-oligo arrays has been successfully assayed in different laboratories. The advantages and disadvantages of this technology are discussed in comparison with other available technologies such as deep sequencing.

## **VALIDATION OF DIAGNOSTIC PROTOCOLS FOR THE DETECTION OF ‘*CANDIDATUS PHYTOPLASMA MALI* AND ‘*CANDIDATUS PHYTOPLASMA PRUNORUM*’**

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The plant pathogens that cause significant economic damage to agricultural crops are subject to phytosanitary regulations aimed at protecting the sanitary and quality of propagative material and production. The availability of official diagnostic protocols, therefore, is necessary for the uniformity of results obtained in different laboratories and to give them a legal value, essential for the application of the phytosanitary measures. Moreover, the entire international community is moving in this direction in order to standardize the diagnosis. The aim of the Italian Project ARNADIA, funded by the Ministry of Agriculture Food and Forestry, was to produce diagnostic protocols officially recognized at a national level for the identification of major plant pathogens. One of the sub-tasks of the ARNADIA Project planned the production of reference and validated protocols for the diagnosis of ‘*Candidatus Phytoplasma mali*’ and ‘*Candidatus Phytoplasma prunorum*’. For this purpose a working group was established, including the main Italian scientific institutions which are involved in the study of both phytoplasmas. Several diagnostic protocols and reagents were compared in five different laboratories, using the same target and no-target reference samples. Protocols of PCR and real time PCR were selected and developed to determine the performance characteristics for validation under the standard UNI CEI EN ISO/IEC 17025 and EPP0 PM7/84 and PM7/98. The results showed that the accuracy and analytical sensitivity of PCR performed with specific primers and nested PCR are comparable, whereas real time PCR results were more reliable. A ringtest among seven Italian laboratories of Phytosanitary Regional Services is currently to evaluate the concordance parameter of all selected protocols.

## **DETECTION OF *PLUM POX VIRUS* IN VARIOUS TISSUES USING FIELD TESTS, SEROLOGICAL AND MOLECULAR TECHNIQUES**

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Intensive testing using fast, reliable and inexpensive diagnostic tools is needed for the successful containment of sharka. We tested various tissues of apricots, peaches and plums infected with *Plum pox virus* (PPV) isolates belonging to PPV-D, PPV-M or PPV-Rec. Field tests (AgriStrip, BIOREBA AG, Switzerland and Immunochromato, NIPPON GENE, Japan), DAS-ELISA (BIOREBA AG, Switzerland), two step RT-PCR and real time RT-PCR were used. At the beginning of the study April flowers and small leaves were sampled. Leaves without symptoms, symptomatic parts of the leaves and asymptomatic parts of the leaves with symptoms were tested at the beginning of May and August. Stalks of symptomatic and asymptomatic leaves were also sampled at the beginning of May as well as dormant buds at the beginning of August. Flowers of apricots and plums in full bloom proved to be a very good source for the detection of PPV. The infection was detected with all the techniques used. Detection in mature leaves using field tests and DAS-ELISA depended on the presence of symptoms. PPV-Was detected with all tested techniques in symptomatic parts of leaves at the beginning of August, but was not detected in asymptomatic leaves using field tests, DAS-ELISA and partly also molecular techniques. PPV-Was detected only in some of the samples of asymptomatic parts of the leaves with symptoms and of stalks by field tests and DAS-ELISA. Reliable detection in buds is very important for testing graftwood. Unfortunately, infection was not confirmed in buds in August using field tests or DAS-ELISA. We conclude that field tests are useful for confirmation of the PPV infection in symptomatic leaves. If symptoms are not present, DAS-ELISA should be combined or replaced by molecular techniques.

## DEEP SEQUENCE ANALYSIS OF VIRAL SMALL RNAS FROM A GREEN CRINKLE-DISEASED APPLE TREE

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### Abstract

Apple green crinkle disease was first discovered in 1934 in Japan. Though it is suspected to be a viral etiology, the causal agent of the disease is unknown. In the search for a virus or viruses associated with green crinkle, we carried out a deep sequence analysis of small RNAs from a green crinkle-diseased apple tree. Low-molecular weight RNAs were extracted from skin tissues of bumpy young fruits of a diseased apple. Small RNAs were separated by polyacrylamide gel electrophoresis and then the libraries of the small RNA populations were constructed for sequencing with an Illumina HiSeq2000 system. Twenty to 26 nt tags were obtained from Illumina reads by cutting off the 3' adapter sequence. A similarity search between these tags and the RefSeq and GenBank virus data was performed by BLASTN analysis. The data disclosed the presence of *Apple stem grooving virus*, *Apple stem pitting virus*, *Apricot latent virus*, *Apple chlorotic leaf spot virus*, *Peach chlorotic mottle virus*, and *Apricot pseudo-chlorotic leaf spot virus* in the diseased apple.

### Introduction

Green crinkle of apple (Kikei-ka in Japanese) was reported in Aomori prefecture, Japan as early as 1934 and confirmed to be transmitted by grafting (Sawamura, 1965). The disease causes bumpy fruit or green crinkle in apple fruits, but no symptoms are evident on leaves, bark, and wood (Desvignes *et al.*, 1999). Though it is suspected to be a viral etiology, the causal virus of the disease is unknown (Howell *et al.*, 2011).

Deep sequencing analysis is a powerful technology to identify all virus species, to reveal the dominant virus species, and also to discover new viruses present in the infected materials (Donaire *et al.*, 2009; Giampetruzzi *et al.*, 2012; Krueze *et al.*, 2009; Pantaleo *et al.*, 2010; Wu *et al.*, 2009). In this study, we analyzed small viral interfering RNAs (vsiRNAs) in an RNA sample from green crinkle-diseased apple fruits using a next generation technology, to determine the viral profile of a green crinkle-diseased apple.

## Materials and Methods

**RNA extraction:** Low-molecular weight RNAs were extracted from pericarp tissues of bumpy young fruits collected in July 2011 (Fig.1) from a diseased apple as follows: Briefly, ca. 2 g of the pericarps were ground into a fine powder in liquid nitrogen using a mortar and pestle, and homogenized with 25 ml extraction buffer (2% [w/v] cetyltrimethylammonium [CTAB], 2% polyvinylpolypyrrolidone [PVP], 100 mM Tris-HCl [pH 8.0], 25 mM EDTA [pH 8.0], 2 M NaCl, 2%  $\beta$ -mercaptoethanol) described by Gasic *et al.* (2004). The homogenates were incubated at 65°C for 15 min and then mixed with 25 ml chloroform for 2 min. After centrifugation, the aqueous phase (24 ml) was ethanol-precipitated, washed with 75% ethanol, and dissolved in 800 $\mu$ l RNase-free water. The solution was mixed with 8 ml Tripure RNA isolation reagent (Roche Diagnostics) and mixed by shaking vigorously for 10 minutes. After adding 1.6 ml chloroform, the solution was shaken vigorously for 1 minute and centrifuged. The ethanol-precipitated total RNA was dissolved in 200 $\mu$ l RNase-free water, one-third volume of 7.5 M LiCl was then added, and it was incubated at 4°C overnight. After centrifugation, the supernatant including small RNAs was ethanol-precipitated. Small RNA pellets were washed with 75% ethanol, and dissolved in 400 $\mu$ l RNase-free water.

**Library preparation and sequencing:** Low-molecular weight RNA fraction which was assumed to contain viral siRNA was subjected to library preparation according to the instructions for small RNA library preparation (Illumina, San Diego, CA), thus providing strand-specific adapter ligation. Briefly, sample RNA was ligated with the 5' RNA adapter, and then with the 3' RNA adapter. Ligated RNA was reverse-transcribed to synthesize the first strand cDNA. PCR was performed using this cDNA as a template to provide and amplify a double strand cDNA library. An amplified product was purified by polyacrylamide gel electrophoresis. This prepared library was analyzed for 51 bp, single read DNA sequences with Illumina HiSeq2000. In total, 189,197,485 reads were obtained.



Fig. 1- Bumpy fruit symptom of Green crinkle-diseased apple (cv. Golden Delicious).

Tab. 1 - Virus- and viroid-related 20-26 nt siRNAs originated from libraries \*

Virus/genus	Green crinkle-diseased apple			
	Reads	%covered length	%(total reads)	%(virus read)
<i>ASGV/Capillovirus</i>	19042	61.2		7.10
<i>ASPV/Foveavirus</i>	11150	74.1		4.16
<i>ApLV/Foveavirus</i>	3015	45.4		1.12
<i>ACLSV/Trichovirus</i>	2157	36.6		0.80
<i>PcCMV/Foveavirus</i>	371	12.7		0.14
<i>ApCLSV/Trichovirus</i>	289	9.6		0.11
<i>CEVd/Pospiviroid</i> **	1089	92.9		0.40
Total virus reads	268321		0.16	
Apple genome	126980571		75.64	
Total	167869270		100	

\*The small RNA sequences were screened by MAQ (version 0.7.1) search for viruses in RefSeq, while the data for CEVd was derived from homology search for GenBank.

\*\**Citrus exocortis yucatan viroid* (GenBank accession number DQ318794) has almost 100% homology with plant mitochondrial rRNA.

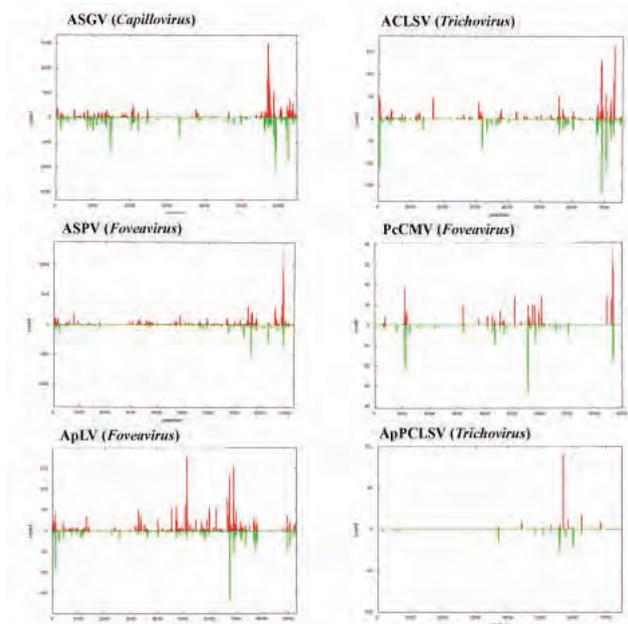


Fig. 2. Distribution of 20~26 nt viral siRNAs along viral genomes. Nucleotide genomic positions occupied by at least one unique read is indicated in red (sense) and green (antisense) bars.

## Results and Discussion

**Analysis of sequencing results:** Some reads contained a part or a whole of the sequence of the 3' RNA adapter at their 3' end region, or a whole of this 3' RNA adapter at their center region. In both cases, sequences over the 5' end of the 3' RNA adapter were taken. After this removal, there were 187,366,394 sequences that had a length of 11 nt to 40 nt. While these sequences derived from a small RNA sample had a variety of nt length, two peaks at 21 and 24 nt for incidence were found, and the major fraction distributing from 20 to 26 nt that contained 167,869,270 sequences (61,210,537 kinds) was further analyzed (1e 1). This fraction of sequences of 20 to 26 nt was mapped to the virus genomes in Refseq and GenBank with MAQ (version 0.7.1).

**Composition of the siRNA populations in a green crinkle-diseased apple:** Though libraries representative of the siRNAs population (from 20 to 26 nt) extracted from bumpy fruits contained 167,869,270 reads (61,210,537 kinds) (Tab. 1), most of the siRNAs (75.64 % of total reads) were derived from apple genome, and only 268321 reads (0.16 % of total reads) appeared to be from virus origins (Tab. 1). In mapping of viral siRNAs (vsiRNAs) to the virus genomes, six known viruses, i.e., *Apple stem grooving virus* (ASGV) (Massart *et al.*, 2011), *Apple stem pitting virus* (ASPV) (Jalkmann and Paunovic, 2011), *Apricot latent virus* (ApLV) (Nemchinov *et al.*, 2000), *Apple chlorotic leaf spot virus* (ACLSV) (Yaegashi *et al.*, 2011), *Peach*

*chlorotic mottle virus* (PcCMV) (James *et al.*, 2007), and *Apricot pseudo-chlorotic leafspot virus* (ApCLSV) (Liberti *et al.*, 2005), were identified (Tab. 1). Among these viruses, ASGV and ASPV are most prevalent species, followed by ApLV, and ACLSV (Tab. 1). The read number of vsiRNA from ASGV was 7.10% and from ASPV was 4.16% out of total virus reads. The reads of ASGV and ASPV covered 61.2% and 74.1% of the viral genomes, respectively (Fig.2). In contrast, the read number of vsiRNA from PcCMV and ApPCMV was low and was 0.14% and 0.11% out of total virus reads, respectively. A homology search of the reads against the GenBank database identified siRNA originating from citrus exocortis yucatan viroid (GenBank accession number DQ318794). The reads were 1089 in number (0.40% of virus reads) and represented 92.9% of the genome (394 nt) of this viroid. However, the sequence (nt no. 15 – 378) of citrus exocortis yucatan viroid has almost 100% homology with plant mitochondrial rRNA. It is reasonable to think that the reads that matched the citrus exocortis yucatan viroid gave rise to apple genome. The aim of this study is to identify the causal virus of apple green crinkle disease. In this research, we found six viruses, ASGV, ASPV, ApLV, ACLSV, PcCMV, and ApCLSV in a green crinkle-diseased apple. ApLV, PcCMV and ApPCMV have not been reported in Japan to date. Unfortunately, the data did not show which virus or viruses found in a diseased apple are the causal agent of a green crinkle-disease. This will be achieved by back-inoculation of infectious clones derived from viruses in a diseased apple. We also did not exclude the possibility that a novel virus may be associated with the disease. Further analysis to identify sequences derived from unidentified viruses is now under study.

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## **VIROIDS: BIOLOGY AND MOLECULAR BIOLOGY OF THE SMALLEST PLANT PATHOGENS**

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Viroids are the causal agents of diseases in plants related to food consumption (potato, tomato, cucumber, hop, coconut, grapevine, subtropical and temperate fruit trees like citrus, avocado, peach, plum apple and pear) and for ornamental purposes (chrysanthemum and coleus). Some viroids are essentially restricted to their natural hosts, but others have expanded host ranges. While certain viroids cause alterations in leaves, stems, bark, flowers, fruits, seeds, and reserve organs as well as delays in foliation, flowering and ripening others have devastating consequences. Some viroids only induce symptoms in a particular organ (bark or fruit), whereas others have more general effects. Infections caused by quite a few viroids result in very mild or no discernible symptoms, due to the lack of symptoms common in naturally infected wild plants that can serve as reservoirs. Symptom expression is favored by high temperature and light intensity, explaining why viroids mainly affect plants grown in warm climates (and in greenhouses), and why curing some viroid infections is refractory to thermotherapy. Vegetative propagation of infected material is the most efficient dissemination route for viroids, and has probably caused certain grapevine and citrus cultivars to contain mixtures of different viroids. Most viroids are also transmitted mechanically and some through seed or pollen. Despite many of the symptoms described above being similar to those incited by viruses, viroids have unique structural, functional and evolutionary features: they are single-stranded, circular RNAs of just 250-400 nucleotides, without protein-coding ability but endowed with autonomous replication. Thus, viroids depend almost entirely on host factors to complete their infectious cycle, and can be essentially regarded as parasites of their host transcriptional apparatus while viruses mostly parasitize the translation machinery of their hosts. Viroids are grouped within the families *Pospiviroidae* and *Awsunviroidae*, whose members replicate in the nucleus and chloroplast, respectively. Viroid replication occurs through an RNA-based rolling-circle mechanism involving the synthesis of longer-than-unit strands catalyzed by host DNA-dependent RNA polymerases (redirected to transcribe RNA templates), processing to unit-length (mediated by hammerhead ribozymes in the family *Awsunviroidae*), and circularization. Following infection, the viroid RNA moves to its replication organelle, with the resulting progeny thus invading adjacent cells through plasmodesmata and reaching distal parts via the vasculature. Viroids are targets of the RNA silencing machinery of their hosts, and some might trigger their pathogenic effect through this route. Finally, viroids most likely have a very ancient evolutionary origin (fulfilling the paradigm that the simplest is the oldest), with the mutation rate estimated for a chloroplast-replicating viroid being the highest reported for any biological entity.

**PEACH LATENT MOSAIC VIROID: DATA SUPPORTING THE INVOLVEMENT OF RNA SILENCING IN PEACH CALICO (EXTREME CHLOROSIS)**

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Peach calico (PC), an extreme chlorosis (albinism) of peach leaves, stems and fruits, is induced by sequence variants of the chloroplast-replicating *Peach latent mosaic viroid* (PLMVd, genus *Pelamoviroid*, family *Avsunviroidae*) harboring a characteristic insertion of 12-14 nt that assumes a hairpin conformation. Here, we show that the primary and not the secondary structure of the inserted hairpin plays a major role in PC, a finding consistent with the hypothesis that PC could be elicited by an RNA silencing mechanism. More specifically, viroid-derived small RNAs (sRNAs) of 21-24 nt generated by the host response against invading nucleic acids, may target for cleavage cell mRNAs thereby triggering a signal cascade that may result in PC. To test this hypothesis, we performed deep sequencing (Illumina) of sRNA libraries from healthy, PC-expressing and latently-infected peach leaves. Then, by semi-quantitative RT-PCR and RNA 3' ligase-mediated rapid amplification of cDNA ends (RACE) we determined that two PLMVd-derived sRNAs, containing the PC-associated insertion and exclusively accumulating in albino tissues, which are a target for cleavage the mRNA encoding the chloroplastic heat-shock protein 90 (cHSP90). These data are the first direct evidence that a viroid may modify the host gene expression of its natural host via RNA silencing. Moreover, since cHSP90 participates in chloroplast biogenesis and plastid-to-nucleus signal transduction in *Arabidopsis*, down-regulation of this protein is consistent with the chloroplast malformations previously reported in PC-expressing tissues, and strongly supports the involvement of RNA silencing in PC. Whether symptom elicitation driven by RNA silencing is a particular case or reflects a general situation in viroid pathogenesis remains to be addressed. Since our study also identified many other peach mRNAs potentially targeted by PLMVd-sRNAs, RNA silencing might have an additional role in plant-viroid interplay, including viroid evolution and adaptation. (A detailed account of these results has been recently published: Navarro *et al.*, *Plant Journal* 70, 991-1003, 2012).

## ***PEACH LATENT MOSAIC VIROID: FURTHER INSIGHTS ON A DETERMINANT ASSOCIATED WITH PEACH MOSAIC***

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*Peach latent mosaic viroid* (PLMVd) displays an unusual pathogenic behavior. While consistent with its name most isolates do not incite foliar symptoms, some incite peach mosaic (PM) or an extreme albinism (peach calico, PC). PLMVd variants inducing PC have a characteristic insertion of 12-13 nt and accumulate in symptomatic leaf sectors but not in the adjacent green sectors. Based on these two findings and on additional experimental data (including deep sequencing of viroid-derived small RNAs) we recently proposed that PC could be mediated by RNA silencing. Extending similar analyses to PM first entails identification of the molecular determinant of PM, a question posing serious difficulties because it is not associated with a specific insertion. Moreover, in contrast with PC which is a well-defined syndrome, PM varies very much in intensity and distribution. After examining different PLMVd-PM isolates over the years, we focused on a few of them. This paper reports results obtained with one of such isolates, recovered from the peach cultivar O'Henry grown in Aragón (Spain), expressing an intense mosaic in leaf sectors intermingled with asymptomatic sectors. Previously, RNAs extracted from both sectors of the same leaves were used to generate by RT-PCR full-length PLMVd-cDNAs that were cloned and sequenced. Their multiple alignment revealed specific changes correlating with symptoms. Two clones (Ab4 and Ab7), differing in a single substitution, were used in order to produce dimeric head-to-tail inserts and then *in vitro* transcripts which were mechanically inoculated onto GF-305 peach seedlings. Intriguingly, while Ab4 incited strong chlorotic symptoms in most leaves, Ab7 only incited mild lesions, providing a first insight of a PLMVd nucleotide directly involved in PM. Moreover, RT-PCR amplification, cloning and sequencing showed that the differential nucleotide substitution was preserved in most of the clones from the symptomatic sectors, but no in those from the surrounding green sectors.

## **DISORGANIZED VIROID DNA FRAGMENT IS TRANSCRIBED IN PLANT**

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Viroids have been adopted as a good model for discovering new biological principles and offering perspectives to continue the advancement of some frontiers of life science. Their disorganized DNA fragment in infectious and has been reported in the viroids of *Potato spindle tuber viroid* (PSTVd), *Hop stunt viroid* (HSVd) and *Peach latent mosaic viroid* (PLMVd). However the counterintuitive property has received poor further support in recent years raising a suspicion about its reliability and generality, and whether the property can extend to similar RNA fragments remains unknown. Identifying the property will provide basic information to document the transcription mechanism for a kind of novel DNA, a disorganized non-coding DNA without integration in plant genomic DNA and lacking protein encoding function. Here we constructed monomeric DNA with blunt ends, monomeric DNA with cohesive ends and dimeric DNA of *Citrus excortis viroid* (CEVd) to infect tomato seedlings, to identify and confirm if they are transcribed using RT-PCR by analyzing their descendant sequences. Results showed that the monomeric DNA with cohesive ends and dimeric DNA of CEVd can recover their transcribed RNA version in the host tissue after they have been inoculated for 20 days. The transcribed RNA was also assessed in terms of its stability, DNA template amount impact on infectivity, cyclization, and biological function. The cohesive ends of monomeric DNA can act as infection helpful factor was also confirmed in PLMVd. We confirmed the novel life phenomenon that a disorganized viroid DNA fragment can be transcribed in plants. It will be helpful to understand the transcription mechanism adopted by plant cells and to develop some protocol to facilitate and simplify genetic transform manipulation by freeing of vector.

## GENERATION OF *IN VITRO* RNA TRANSCRIPTS AND INFECTIOUS FULL-LENGTH cDNA CLONES OF ASPV AND ASGV

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In commercial apple cultivars infection with viruses causes up to a 60% loss in yield. *Apple stem pitting virus* (ASPV), *Apple stem grooving virus* (ASGV), and *Apple chlorotic leaf spot virus* (ACLSV) are the three most common latent viruses in apple worldwide. These viruses are highly variable and infect several woody host plants with different symptoms. Infectious cDNA clones provide the opportunity to study pathogenicity and symptomatology of a determined variant of a virus. Two strategies for the generation of an infectious full - length cDNA clone of the ASPV were attempted. Initially a ligation strategy was attempted by subdividing the genome of ASPV isolate PB 66 into three fragments. These were ligated into the plasmid p1657 containing the 35S promoter. Due to an incomplete 5'-end of the sequence the resulting cDNA clones showed no infectivity on different host plants. The second strategy was based on PCR of the full length of ASPV and ASGV. Comparison of the 5' – and 3' end of different ASPV isolates showed highly conserved domains which were used as primers for PCR. Generation of infectious *in vitro* RNA transcripts of ASPV and ASGV were obtained by the addition of the T7 promoter sequence to the forward primers of full-length PCR fragments. *In vitro* RNA transcripts of ASGV infected 5 out of 6 mechanically inoculated *N. occidentalis* 37B plants, whereas transcripts of ASPV infected only 4 out of 42 tobacco plants. The Circular Polymerase Extension Cloning method (CPEC) was used to generate an infectious full – length cDNA clone of ASPV and ASGV in pBin V297. *N. occidentalis* 37B plants were infected by inoculation with *Agrobacterium tumefaciens* containing the pBin vector with the full- length cDNA clone for both viruses. The infection rates were 3% for ASPV and 22% for ASGV.

## GENOME ORGANIZATION OF *CHERRY LEAF ROLL VIRUS* AND ANALYSES OF FUNCTION OF VIRUS-ENCODED PROTEINS

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*Cherry leaf roll virus* (CLRV) infects many stone and some small fruits. Recently, the complete genome was determined revealing the genome organization of the bipartite positive stranded plant RNA virus (*Secoviridae* family, genus *Nepovirus*). Our aim now is to characterize the functions of CLRV-encoded proteins. As a prerequisite, it is necessary to understand the processing of mature proteins from polyproteins encoded by vRNA1 (P1) and vRNA2 (P2). The viral proteinase and putative cleavage sites will be analyzed *in vitro* in order to determine the specific processing sites utilized during proteolytic processing of P1 and P2. CLRV is transmitted by pollen and seeds. Systemic infection of a host plant is achieved by cell to cell movement via plasmodesmata and long-distance transport through the vascular system. Members of the family *Secoviridae* are transported as virions, thus requiring the coat protein (CP). The viral movement protein (MP) inducing tubular structures by multimerization within plasmodesmata is necessary for the passage of virus particles to adjacent cells. Virus-like particles (VLPs) have been observed within tubules in anther cells and pollen grains of CLRV-infected birch and walnut. However, the underlying interactions of CLRV-CP and MP involved in cell to cell movement have never been investigated. We applied the yeast two-hybrid system (YTHS) to address this question. Dimerization of the MP (385 aa, 42 kDa) and the CP (512 aa, 54 kDa) was shown as well as the specific protein interaction of both viral proteins. Additionally, binding of the viral MP to a plant protein (At-4/1) which is localized at plasmodesmata was demonstrated. At-4/ was shown to interact with the tubuli-forming MP of *Tomato spotted wilt virus* (TSWV) and facilitates intra- and intercellular trafficking. This suggests that CLRV and TSWV are utilizing the same cell to cell transport mechanism in their host plants.

## **GENOME SEQUENCE VARIABILITY OF A *PLUM POX VIRUS* ISOLATE RELATED TO ITS LONG-TIME MULTIPLICATION IN DIFFERENT HOST SPECIES**

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To study spontaneous adaptations of the *Plum pox virus* (PPV) to various *Prunus* host species, the PPV-M isolate VAR-2 (originally isolated from peach) was chip-budded on peach, apricot and plum trees in 2006. Five years later the virus was isolated from all these sources to perform the complete genome sequencing. Obtained subisolate sequences were compared to the VAR-2 sequence determined from the original source, as well as from its passage in *Nicotiana benthamiana*. PPV-M tends to infect mainly peaches under field conditions and we presume that this host preference is genetically determined. Therefore long-term-maintenance in atypical host species could lead to selection and fixation of mutations which would overcome this limitation and escape the original host preference. Results of sequence comparisons will be presented, together with conclusions outlining the factors affecting specific host-PPV interactions.

## SCREENING OF TURKISH LOCAL APRICOT VARIETIES AND CROSSINGS BY MOLECULAR MARKERS LINKED TO *PLUM POX VIRUS* RESISTANCE

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Turkey is the most important producer and exporter country of apricot, *Prunus armeniaca*, which is an important temperature fruit. The production of apricots for fresh market relays on foreign cultivars grown on Mediterranean and Aegean regions while Malatya is the most important region for production of dry apricot but base on a several local varieties. The sharka diseases caused by the *Plum pox virus* (PPV) has been reported in several parts of Turkey, although there are no reports for its presence in Malatya. However, when today's production conditions are considered, it may be assumed that it is just a matter of time for the PPV to reach Malatya. Because of that, breeding PPV resistant cultivars having the pomological characteristics of local varieties became an important objective from an economically point of view. In order to breed new cultivars resistant to PPV, marker assisted selection based on markers linked to PPV resistance were used for screening 19 local apricot genitors and progenies obtained from crossings between the PPV resistant cv. Stark Early Orange (SEO) and local cvs. such as Hacıhaliloğlu, Kabaası, Hasanbey, Çöloğlu, Adilcevaz5, Şekerpare and Mahmudun Eriği. The markers PGS1.21 and PGS2.23 co-segregating with resistance to PPV-Were used to screen a total of 90 apricot progenies. None of the local genitors had alleles linked to PPV resistance. Among the progenies screened, two seedlings from Hacıhaliloğlu by SEO, seven from Kabaası by SEO, three from Hasanbey by SEO, six from Çöloğlu by SEO, three from Adilcevaz5 by SEO, nine from Şekerpare by SEO and one from Mahmudun Eriği by SEO presented resistant alleles.

***APPLE LATENT SPHERICAL VIRUS VECTORS FOR CROSS PROTECTION AGAINST ZUCCHINI YELLOW MOSAIC VIRUS***

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*Apple latent spherical virus* (ALSV), classified into a genus *Cheravirus*, was originally isolated from an apple tree in Japan. The virus can experimentally infect a broad range of plant species including cucurbits and legumes without causing symptoms. We previously reported that ALSV-based vectors could effectively induce stable virus-induced gene silencing (VIGS) for long periods in plants. Here, we show that a genetically engineered ALSV vector containing a partial genome sequence of *Zucchini yellow mosaic virus* (ZYMV) display a high degree of cross protection against ZYMV. A wild-type ALSV and a ALSV vector containing ZYMV sequence (ca. 200 bp) were inoculated to the cotyledons of cucumber. Five to ten days after inoculation, the infected plants were challenged with ZYMV expressing GFP. Protection was evaluated by symptom development on plants and fluorescence microscopy and ELISA monitored the accumulation of the challenging viruses. The results showed that no symptoms were observed on any plants infected with the ALSV vector containing the viral genome sequence for a month after challenge inoculation. The accumulation of ZYMV was strongly reduced in the plants. Thus, it is thought that the infection of the ALSV vector containing ZYMV genome sequences induced VIGS to target the ZYMV genomic RNA, resulting in strong cross protection against ZYMV.

## **STRAWBERRY MARGINAL CHLOROSIS (SMC): A DISEASE OF VARIOUS BACTERIAL AND VIRAL ETIOLOGIES IN FRANCE**

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Marginal chlorosis has been observed on strawberry plants in France since 1988. Previous studies have demonstrated the association of two phloem restricted bacteria with the disease: '*Candidatus* Phlomobacter fragariae' and Stolbur phytoplasma. The improvements in detection techniques as well as the discovery of two new phloem-restricted bacteria associated with SMC; '*Candidatus* Phytoplasma fragariae' in Lithuania and the gamma 3 proteobacterium previously known as the agent of 'basses richesses' syndrome of sugar beet (SBRp) in Italy led us to reassess SMC etiology in France. Two specific triplex real time PCRs were engineered to detect the strawberry mitochondrial *cox* gene as internal control in addition to Stolbur phytoplasma and '*Ca. Phlomobacter fragariae*' in the first test, and the detection of '*Ca. Phytoplasma fragariae*' and SBRp in the second test. SMC surveys were carried out in different locations in France in production fields as well as in nurseries. Phloem-restricted bacteria associated with typical SMC were detected using the two triplex real time PCR and additional tests were carried out to also detect the *Strawberry mild yellow edge virus* (SMYEV), a potexvirus also associated with strawberry yellows. Stolbur phytoplasma was again more frequently detected in SBRp and in production fields, Phlomobacter fragariae was prevalently detected in production fields. SBRp and '*Ca. Phytoplasma fragariae*' were associated with 1-5 % of the disease cases. SMYEV was detected in nurseries and in production fields with incidences between 13 to 30%. SMYEV coat protein sequences revealed a significant genetic diversity of SMYEV in the French strawberry production.

## IDENTIFICATION AND PHYLOGENETIC ANALYSIS OF VIRUSES INFECTING STONE FRUITS AND STRAWBERRY IN EGYPT

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### Abstract

A total of 102 samples of *Prunus persica* (peaches), *P. armeniaca* (apricot) and *P. domestica* (plums) and 50 samples of *Fragaria X ananassa* (strawberry) were collected from commercial fields and nurseries in Alexandria, El Beheira and El Menofia governorates in Egypt in 2009/2010. Sample collection was based on virus diagnostic symptoms on different identified cultivars. DAS-ELISA and RT-PCR were used to identify infection by *Plum pox virus* (PPV), *Prune dwarf virus* (PDV), *Prunus necrotic ringspot virus* (PNRSV), *Apple chlorotic leafspot virus* (ACLSV), *Apple mosaic virus* (ApMV) and *Strawberry latent ringspot virus* (SLRSV) in stone fruits. However, strawberry samples were tested for *Strawberry vein banding* (SVBV), *Strawberry crinkle virus* (SCV), *Strawberry mild yellow edge virus* (SMYEV), *Strawberry latent ringspot virus* (SLRSV) and *Strawberry mottle virus* (SMoV). In stone fruits only PPV, PDV and SLRSV were detected, whereas SMYEV was the only virus identified in all tested strawberry samples. RT-PCR identified more infected stone fruit samples than ELISA due to its higher sensitivity. However, nRT-PCR identified more SLRSV-infected samples than RT-PCR and ELISA. nRT-PCR identified more infected strawberry samples than RT-PCR and ELISA. PCR amplification of different amplicons from the detected isolates allowed partial sequences and phylogenetic analysis of different isolates of PPV (13), PDV (7), SLRSV (4) and SMYEV (2). The four Egyptian SLRSV isolates were highly distinct from the other mentioned isolates reported in GenBank (7-73%). Therefore, they are thought to belong to a new strain of SLRSV. This study indicates infected cultivars and illustrates the evolutionary relationship between the Egyptian viral isolates identified and compares them to other reported isolates.

## Introduction

Plant pathogens cause significant damage to crops with at least 10% of global food production being lost due to diseases (Strange and Scott, 2005). This may be due to importing or local exchange of plant material for propagation or commercial growing of these fruits that can be infected by a wide range of viruses that may cause damage to affected crops and result in significant economic losses to the agricultural industries (Zhang *et al.*, 2010). Stone fruits are very important in the agricultural economy of many countries. Stone fruit trees are a major fruit industry in the Mediterranean area (El-Maghraby *et al.*, 2007). The stone fruit cultivated area in Egypt is 49,209 ha with an approximate yearly production of 476849 tons, 6 % of total fruit production, (El Maghraby *et al.*, 2007). The total stone fruit area is made up of the old land (16 % with production of 86.933 tons/year) and new reclaimed land (84 % with production of 389.916 tons/year). The production areas are concentrated in north and northeastern parts of the country. They are cultivated in many governorates, especially: Giza, El-Fayoum, Beni-Sweif, El-Qualubia, El-Menofia, El Gharbia, El-Sharkia and North Sinai (El Maghraby *et al.*, 2007). Stone fruit trees are hosts to a large number of economically important and common RNA plant viruses that exhibit very different biological properties as well as structural characteristics, genome expression strategies and can cause substantial economic losses (Sanchez-Navarro *et al.*, 2005). They belong to different genera, the most frequent occurring in the genera: *Potyvirus*: Plum pox virus (PPV); *Illarvirus*: Prune necrotic ring spot virus (PNRSV), Prune dwarf virus (PDV), Apple mosaic virus (ApMV) and *Trichovirus*: Apple chlorotic leaf spot virus (ACLSV). Strawberry latent ringspot virus (SLRSV) (genus *Sadwavirus*), Tomato ringspot virus (ToRSV) and Peach rosette mosaic virus (PRMV) (genus *Nepovirus*) are generally less frequently detected (Myrta *et al.*, 2003). All these viruses, alone or in combination, can affect fruit yield, fruit maturity and tree growth (Youssef and Shalaby, 2009).

Strawberry (*Fragaria* spp.) is one of the most economically important small fruit crops in Egypt (El-gaied *et al.*, 2008). According to the Egyptian Agricultural Statistics (2004) the total cultivated area of strawberry is 8,677 Faddens, giving a yield of 99,091 tons. It is an important commercial fruit grown in different parts of the country, with a great potential for export if virus-free planting material is employed to increase production and maintain quality. Many viruses form a major threat for the strawberry industry in Egypt, causing severe economic losses. There are four main aphid-borne viruses infecting strawberry worldwide: *Strawberry crinkle virus* (SCV), *Strawberry mild yellow edge virus* (SMYEV), *Strawberry mottle virus* (SMoV) and *Strawberry vein banding* (SVBV) (Thompson and Jelkmann, 2003).

To date, the only effective way to prevent virus spread in stone fruit and strawberry crops is through the use of healthy material for plantations. Therefore, great efforts have been made during recent years to improve the sensitivity and speed of molecular diagnostic methods. In this study, we describe the development and optimization of the detection of viruses affecting stone fruit trees (ACLSV, PDV, PNRSV, PPV, ApMV and SLRSV) and strawberry (SCV, SVBV, SMYEV, SMoV and SLRSV) by molecular techniques, in addition to partial sequence identification of the Egyptian isolates of detected viruses.

## Materials and Methods

**Field surveys and ELISA test:** A survey and collection of samples from different commercial or local orchards and nurseries of peaches (*Prunus persica*), apricot (*Prunus armeniaca*) and plum (*Prunus domestica*) was conducted. Selected governorates of Egypt namely: El-Beheira (El Nobarria), El-Menofia and Alexandria, representing old, reclaimed and recently reclaimed land were visited during the winter or flowering season (from November to December) of 2009 and spring to early summer season (from mid April to June) of 2010. The aim the survey was the detection and assessment of the incidence of major stone fruit viruses (ACLSV, PDV, PNRSV, PPV, ApMV and SLRSV). Leaf samples including the petioles and midribs showing various types of virus and virus-like symptoms related to PPV and PDV disease symptoms were collected for laboratory testing. A total of 102 samples, of which 72 peaches (representing cultivars Nemaguard, Swelling, Florida Prince and Desert Red), 12 apricots (representing cultivars Balady and Canino) and 18 plums (representing cultivars Hollywood, Santa Rosa, Golden Japanese and Kelsey) were collected. A survey and collection of samples from different commercial strawberry (*Fragaria x ananassa*) fields and nurseries in the El-Beheira governorate of Egypt, representing reclaimed land (Bader centre) and recently reclaimed land (El-Nobarria), was conducted during the fall season (from September to November) of 2009 and during spring to early summer season (from mid March to May) of 2010. The aim of the survey was the detection and assessment of the incidence of major strawberry viruses (SCV, SVBV, SMYEV, SMoV and SLRSV). Leaf samples showing various types of virus and virus-like symptoms related to strawberry viruses were collected for laboratory testing. A total of 50 strawberry samples, representing different cultivars such as Sweet Charlie, Florida, Camarosa, Festival and Rosa Linda were collected. All samples were labeled, wrapped in plastic bag and stored at 4°C until use for laboratory analysis. Double Antibody Sandwich (DAS-ELISA) according to Clark and Adams (1977) as modified by Lister and Rochow (1979); Weiss and Van Regenmortel (1989) was applied. ELISA commercial kits (BIOREBA, Switzerland) were used according to the manufacturer's instructions. All samples, whose absorbance values were at least twice the absorbance values of the healthy control, were considered positive. Positive reactions were confirmed by using the polymerase chain reaction (PCR) with primers specific for each virus.

**Preparation of total RNA extracts:** Total RNA was extracted from young leaf samples using the CTAB RNA EXTRACTION METHOD according to the procedure described by Chang *et al.* (1993) for the detection of stone fruit viruses. Total nucleic acid extraction obtained by a modified CTAB method (Chang *et al.*, 2007), total RNA extraction obtained by the modified CTAB method (Chang *et al.*, 1993) or by a commercially available plant RNeasy kit (GE Healthcare, UK) were compared for the detection of the strawberry viruses. The RNA pellet was resuspended in 100µl of nuclease-free water and stored at -20°C until required.

**RT-PCR analyses:** Synthesis of first-strand cDNA of PPV-Was performed using ImProm-II Reverse Transcriptase (Promega, USA). 1 µl of reverse primer (5 nmol/ml) and 1 µl total RNA were added to 3 µl of nuclease-free water. The mixture was incubated at 70°C for 5 min then for 10 minutes on ice. After incubation, the mixture was added to 15 µl ImProm-II reverse transcriptase mix containing 4 µl of 5x ImProm-II buffer (Promega), 1.2 µl of 25mM MgCl<sub>2</sub>, 1 µl of 10 mM dNTP mix (Promega), 1 µl of 0.1 µM DDT, 0.5 µl of 40u/µl RNase inhibitor, 6.3 µl of H<sub>2</sub>O and 1 µl of ImProm-II reverse transcriptase (Promega). The mixture was incubated at 25°C for 5 min then at 42°C for 1 h. Synthesis of first-strand cDNA of PDV, ApMV, PNRSV and ACLSV was performed by Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) (Promega, USA) in the presence of random primer (Roche). 1 µl of total RNA was added to 4 µl reverse transcriptase mix containing 1 µl of 5x M-MLV buffer (Promega), 0,5 µl of 5 nmol/ml random primer (Roche), 0,5 µl of 10 mM dNTP mix (Promega) and 50 U of M-MLV reverse transcriptase (Promega). The mixture was incubated at 37°C for 1h. Synthesis of first-strand cDNA of SLRSV was performed by Avian Myeloblastosis Virus (AMV) Reverse Transcriptase (Promega, USA). 1 µl total RNA was added to 4 µl reverse transcriptase mix containing 1 µl of 5x AMV buffer (Promega), 1 µl of 5 nmol/ml reverse primer, 0,5 µl of 10 mM dNTP mix (Promega) and 50U of AMV reverse transcriptase (Promega). The mixture was incubated at 48°C for 1h. Viral cDNAs were amplified by PCR in a reaction mixture (25 µl final volume) containing 5x Mg-free Buffer (Promega), 3,0 µl of 25 mM MgCl<sub>2</sub> (Promega), 1 µl of 5 nmol/ml solution of each primer, 0,5 µl of 10 mM dNTP mix (Promega), 1.25 U of Taq DNA polymerase (Promega) and 5 µl of the RT mixture. Regarding SMYEV, SMoV, SVBV and SCV, 1 µl of total RNA or total nucleic acid (used as a template in RT in order to synthesize cDNA) was added to a one step RT-PCR reaction mixture (10 µl final volume) containing 5x Mg-free Buffer (Promega), 1 µl of 25 mM MgCl<sub>2</sub> (Promega), 0,5 µl of 5 nm/ml of each primer, 0,3 µl of 10 mM dNTPs (Promega), 0,25 U of Taq DNA polymerase (Promega) and 12 U of M-MLV reverse transcriptase (Promega).

**N-PCR analyses:** Regarding SLRSV, RT-PCR was followed by nested PCR; 200 µl of H<sub>2</sub>O was added to the RT-PCR product. After that, 1 µl of diluted RT-PCR product was added to a n-PCR reaction mixture (25 µl final volume) containing 5x Mg-free Buffer (Promega), 2 µl of 25 mM MgCl<sub>2</sub> (Promega), 1 µl of 5 nmol/ml of each primer, 0,5 µl of 10 mM dNTP mix (Promega) and 0.25 U of Taq DNA polymerase (Promega). Regarding SMYEV, *SCV* and *SMoV*, RT-PCR was followed by nested PCR; 10 µl of RT-PCR product was added directly to a n-PCR reaction mixture (25 µl final volume) containing 5x Mg-free Buffer (Promega), 2,5 µl of 25 mM MgCl<sub>2</sub> (Promega), 1 µl of 5 nm/ml of each primer, 0,5 µl of 10 mM dNTP mix (Promega) and 0.25 U of Taq DNA polymerase (Promega). The PCR system (Gene Amp 9700 thermocycler, Applied Biosystem (ABI), USA) was used for performing PCR amplification. Details such as type of PCR test, specific synthetic oligonucleotides as primers used, region amplified, expected product size and reference for each test used to detect stone fruit viruses under investigation are shown in Tab. 1 and 2. Optimal reaction conditions, such as incubation times and temperatures during PCR amplification, were evaluated for each virus as shown in Tab. 3 and 4. The PCR products (10 µl) were analysed by

gel electrophoresis on a 1% agarose gel and visualized on a UV-transilluminator after staining with ethidium bromide. The 1-kb DNA ladder (Promega) was included to determine the size of amplified products.

**Cloning, sequencing and phylogenetic analyses:** The RT-PCR products were purified using Wizard SV Gel and Clean-Up System Kit (Promega, USA) according to the manufacturer's instructions, ligated into pGEM-T Easy vector (Promega) and transformed into *Escherichia coli* strain M1022. Recombinant plasmids containing inserts of the expected size, identified by direct PCR amplification, were purified using a Wizard plus SV miniprep kit (Promega). The identity of the plasmid insert was verified by sequencing (MWG-Biotech AG, Germany) and the sequences obtained were submitted to GenBank. Computer-based comparison of partial nucleotide sequences of the Egyptian viral isolates and corresponding genome regions of other geographical isolates present in GenBank, were generated by multiple sequence alignments using Workbench version 3.2, CLUSTALW program (Thompson *et al.*, 1994). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4, (Bootstrap test of phylogeny (1000 replicates), Neighbor-Joining method) (Tamura *et al.*, 2007).

Tab. 1 - Molecular methods used for the detection of stone fruit viruses.

Virus	Test	Primer name	Primer sequence (5'-3')	Region amplified	Expected product size (bp)	Reference
PPV	RT-PCR	Schneider F	CCAATAAAGCCATTGTTGGATC	Partial coat protein & the 3' UTR	593	Chen and Adams, 2001 & Scheider <i>et al.</i> , 2004
		M4T	GTTTTCCAGTCACGAC(T)15			
PDV	RT-PCR	PDV 2F	ATCGAGGGATGGATG-GATAAAAATAG	Partial coat protein & the 3' UTR	204	Candresse <i>et al.</i> , 1997
		PDV IR	TAGTGCAGGTTAACCAAAAAG-GAT			
PNRSV	RT-PCR	ilar2 F	CCACCCGAGAGGTTGGCA	Partial coat protein	206	Candresse <i>et al.</i> , 1997
		ilar1 R	TTCTAGCAGGTCTTCATCGA			
ACLSV	RT-PCR	ACLSV F	GGCAACCCTGGAACAGA	Partial coat protein	358	Nemichov <i>et al.</i> , 1995
		ACLSVR	CAGACCCCTTATTGAAAGTCGAA			
ApMV	RT-PCR	ApMV F	ATCCGAGTGAACAGTC-TATCCTCTAA	Partial coat protein	262	Menzel <i>et al.</i> , 2002
		ApMV R	GTAACCTCACTCGTTATCACG-TACAA			
SLRSV	RT-PCR	3DR	AGGCTCAAGAAAACACAC	Partial coat protein	739	Ratti <i>et al.</i> , 2008
		F1	GGCTGATCCTCGTGAGAC			
n-PCR	n-PCR	2R2	GCGTGTGGCGTCGCTAAT	Partial coat protein	203	
		5DF	CCCTTGGTTACTTTTACCTCCT			

Tab. 2 - Molecular methods used for the detection of strawberry (*Fragaria x ananassa*) viruses.

Virus	Test	Primer name	Primer sequence (5'-3')	Region ampli- fied	Expected product size (bp)	Reference
SVBV	RT-PCR	SVBV F	AACCTATCGTCCGAACC	Partial coat protein	670	Ratti <i>et al.</i> , 2008
		SVBV R	TCAAAAGCCTACATGGAGAAA			
SCV	RT-PCR	SCV F2	GGACTGTAATGTCACCAGAG	Partial coat protein	1253	Ratti <i>et al.</i> , 2008
		SCV R2	CCTTTGCTTCCTTCTGCTG			
	SCV 1FW	ACTGTAATGTCACCAGAGAAG				
	SCV 1RV	TTCTGACACTAGTAGAIGTCC				
SMoV	RT-PCR	SMoV F1	AGGGAAACCCTGGTAGTCC	Partial coat protein	581	Ratti <i>et al.</i> , 2008
		SMoV R	ATTCGGTTCACGTCCTAGTCTCAC			
	SMoV F	TAAAGGCCACGACTGTGACAAAAG				
	SMoV R3	CAAAACAGTTAAATGTCATGATGG				
SLRSV	RT-PCR	3DR	AGGCTCAAAGAAAACACAC	Partial coat protein	739	Ratti <i>et al.</i> , 2008
		F1	GGCTGATCCTCGTGAGAC			
	2R2	GCGTGTGGCGTCGCTAAT				
	5DF	CCCTTGGTTACTTTTACCTCCT				
SMYEV	RT-PCR	SMYEV R2	(T)15AAGGAAAAAGAAAAACAAC	Partial coat protein	812	Ratti <i>et al.</i> , 2008
		SMYEV F3	GTCGAATCCATTGCCAAT			
	SMYEV R3	GGATCTACTAGTCTCCTTTAGTCAGG				
	SMYEV F4	GCTCTGATATGTTCTACGCC				
	n-PCR				292	

Tab. 3 - Thermal cycling conditions tested for the optimization of PCR protocols for the detection of stone fruit viruses.

Primer name	Initial denaturation step	Number of cycles	Denaturation step	Annealing step	Elongation step	Final extension step
Schneider/ M4T	94°C /1 min	(20,25,30&35)	94°C /30s	(43,47,52&55°C)/ 1 min	72°C / (45s, 1 & 2 min)	72°C /10min
PDV2F/ PDV1R	94°C /2 min	(20,25,30&35)	94°C /30s	(40,45,50&56°C)/ 30s	72°C / (45s, 1&1,5 min)	72°C /7 min
ilar2 F/ ilar1R	94°C /2 min	(20,25,30&35)	94°C /30s	(40,45,50&56°C)/ 30s	72°C / (45s, 1 & 1,5 min)	72°C /7 min
ACLSV F/ ACLSV R	94°C /5 min	(30,35,40&45)	94°C /20s	(45,50&56°C)/ 20s	72°C /1 min	72°C /10 min
ApMV F/ ApMV R	94°C /2 min	(30,35,40&45)	94°C /10s	(45,50, 55 & 60°C)/ 10s	72°C / 1 min	72°C /10 min
3DR/ F1	94°C /1 min	(25,30&35)	94°C /10s	(45,50&54°C)/ 10s	72°C /45 s,	72°C /2 min
2R2/ 5DF	94°C /1 min	(25,30,35&40)	94°C /10s	(52,54,58&60°C)/10s	72°C /1 min	72°C /2 min

Tab. 4 - Thermal cycling conditions tested for the optimization of PCR protocols for the detection of strawberry (*Fragaria x ananassa*) viruses.

Primer name	Initial denaturation step	Number of cycles	Denaturation step	Annealing step	Elongation step	Final extension step
SVBV F/R	94°C /1min	(20,25,30&35)	94°C /10s	(45,50&54°C)/10s	72°C /1 min	72°C /1 min
SMoV F1/R	92°C /5min	(18,20&25)	94°C /35s	(45,50&55°C) /35s	72°C /1 min	72°C /1 min
SMoV F/R3	94°C /2 min	(30,35&40)	94°C /10s	(45,52,55°C) /10 s	72°C / 25s	72°C /1min
SCV F/R2	94°C /5 min	(18,20&25)	94°C /35s	(58°C) /35s	72°C / 1 min	72°C /1 min
SCV IFW/IRV	94°C /5 min	(30,35&40)	94°C /10s	(45,50,52&55°C) /10s	72°C / (45s, 1& 2 min)	72°C /1 min
SMYEV R2/ F3	94°C /5 min	(18,20&25)	94°C /35s	(40,45,50&55°C) / 35s	72°C / (40s, 1&1.5 min)	72°C /1 min
SMYEV R3/ F4	94°C /5 min	(20,25,30&35)	94°C /10s	(40,45,50&55°C) / 10s	72°C / 25s	72°C /1 min
3DR/ F1	94°C /1 min	(30,35,40&45)	94°C /10s	(52,54,58&60°C) / 10s	72°C / (45 s, 1 & 2 min)	72°C /2 min
2R2/ 5DF	94°C /1 min	(30,35&40)	94°C /10s	(52,54,58&60°C) /10s	72°C /1 min	72°C /2 min

## Results

**Virus symptoms observed in orchards:** During the period of the survey peach, plum and apricot trees showing characteristic symptoms known to be associated with PPV-Were detected. However, characteristic symptoms known to be associated with PDV were only observed on peach trees. For PPV, on plums, leaf symptoms included pale green spots, rings and line patterns as shown in Fig.1A. On apricots, symptoms were in the form of pale green or chlorotic rings and blotches on leaves Fig.1B. Leaves of infected peaches showed small chlorotic blotches and distortion reported in Fig.1C. For PDV, the symptoms were detected only on peach trees, no characteristic symptoms were observed on apricot and plum trees of the visited orchards. Peach plants exhibit mild to severe stunting and shortening of internodes, rosette formation in the developing shoots, reduction in plant growth and fruit production as shown in Fig 2. The major observations in the survey were that the distribution of infected trees in visited orchards was scattered, symptoms were observed on leaves from the base and middle part of the shoots, while the younger leaves close to the tip part did not show any symptoms. The infestation of unidentified aphids was common in most surveyed orchards. Regarding the strawberry plants virus-like symptoms were observed as shown in Fig.3. The symptoms were seen on the older and new leaves. Older leaves were rolled upward (cupped) and displayed purple discoloration Fig.3A; new leaves showed size reduction, deformations, shortened petioles and were generally cupped; the leaflets also exhibited cupped, dwarfed, asymmetrical and pale green foliage with typical yellow or chlorotic margins Fig.3B,C and D. The distribution of infected plants in the visited fields was mainly observed in the form of patches. The infestation of unidentified aphids was common in most surveyed plantations.

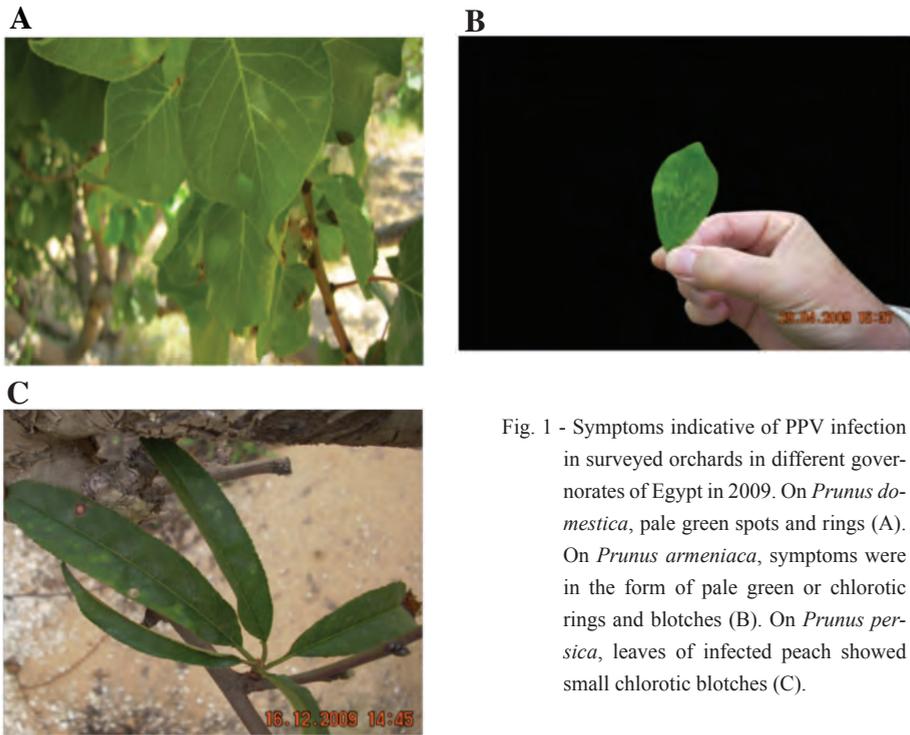


Fig. 1 - Symptoms indicative of PPV infection in surveyed orchards in different governorates of Egypt in 2009. On *Prunus domestica*, pale green spots and rings (A). On *Prunus armeniaca*, symptoms were in the form of pale green or chlorotic rings and blotches (B). On *Prunus persica*, leaves of infected peach showed small chlorotic blotches (C).

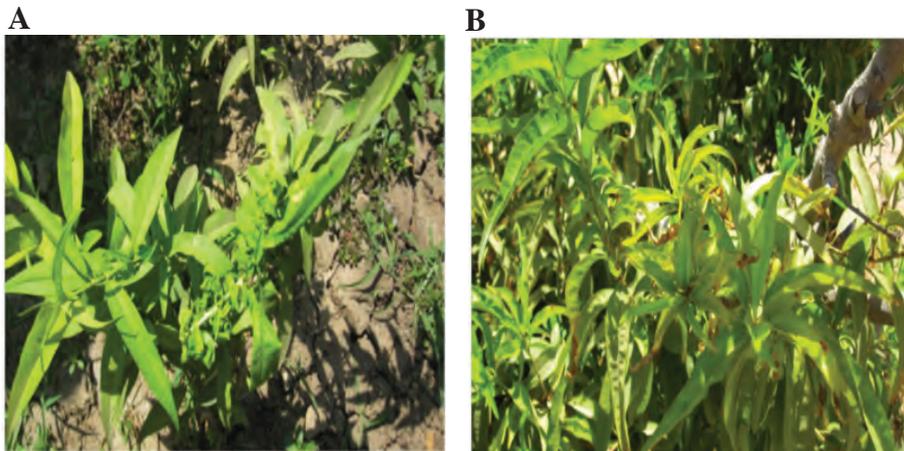


Fig. 2 - Symptoms indicative of PDV infection in surveyed orchards in different governorates of Egypt in 2009. On *Prunus persica*, infected plants showed severe stunting of internodes, rosette formation in developing shoots (A and B).

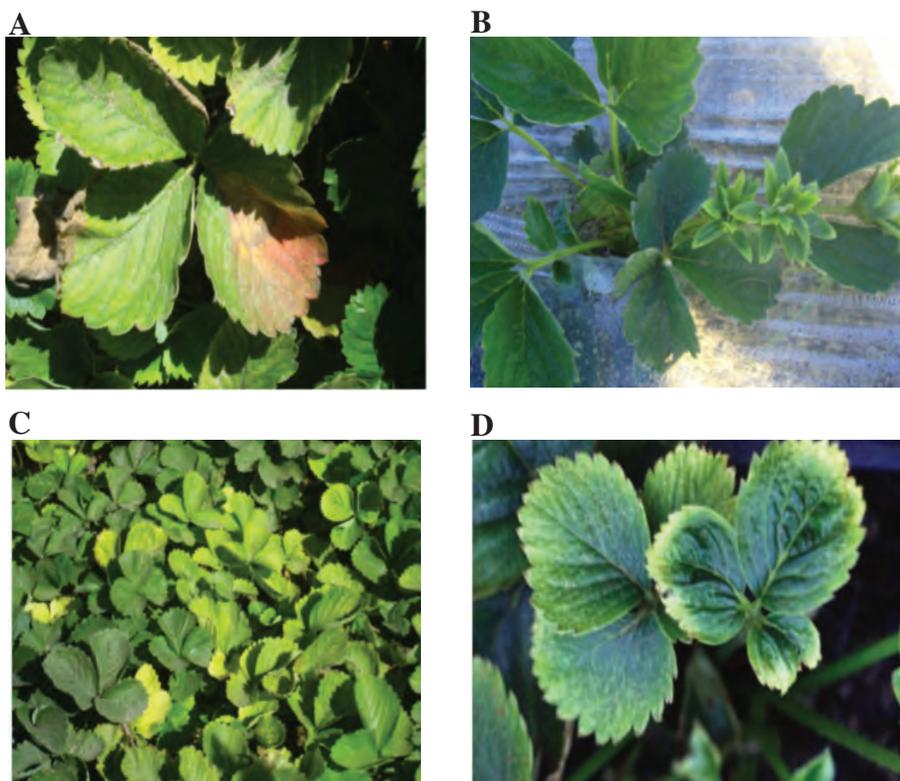


Fig. 3 - Symptomatic strawberry (*Fragaria x ananassa*) plants observed in selected commercial fields in El-Beheira governorate during 2009. Older leaves were rolled upward (cupped) and displayed purple discoloration (A); new leaves showed size reduction, deformations, shortened petioles, yellowing and marginal chlorosis (B); and the leaflets exhibit cupped, dwarfed, asymmetrical and pale green foliage with typical yellow or chlorotic margins (C and D).

**ELISA for detection of stone fruit viruses:** A total of 102 symptomatic leaf samples, from different species of stone fruits, were collected and tested using DAS-ELISA to confirm the presence of PPV, PDV, PNRSV, ACLSV, ApMV and SLRSV infection in collected samples. Results indicated that the overall average infection level was (20.6%, 21 out of 102). 18.6% of the samples were infected with one virus and 2% were infected with more than one virus (mixed infection). The detected viruses were PPV (in 16 out of 102 samples, 15.7%), PDV (6/102, 5.8%) and SLRSV (1/102, 0.98%). No infection by PNRSV, ACLSV and ApMV was detected in the collected samples. PPV-Was the most prevalent virus whereas double infection of PPV and PDV was detected in 1.96% of the total samples of plum and PDV-SLRSV mixed infection in the 0.98% of peach samples tested. The highest incidence was recorded in El-Menofia governorate (45%), followed by Alexandria (41%) and by El-Beheira (23%). The infection level of different species was: 50% (9/18) in plum, 16.6% (2/12) in apricot and 13.8% (10/72) in peach.

**ELISA for detection of strawberry viruses:** A total of 50 symptomatic strawberry leaf samples were collected and tested using DAS-ELISA to confirm the presence of SMYEV and SLRSV infection in Egypt. From a total of 50 samples, SMYEV infection was detected in 8 samples. Results obtained showed that the total average of SMYEV infection was 6% (3/50). Within the collected samples, the infection percentage in strawberry cultivars Sweet Charlie, Florida, Camarosa, Festival and Rosa Linda in El-Beheira was 0% (for the first three cultivars), 14.2% and 12.5%, respectively. The highest incidence was recorded in reclaimed land (Bader centre) (6.6%) followed by recently reclaimed land (El-Nobaria) (5%). Negative reactions were observed with all the healthy controls. Results showed that the occurrence of SMYEV infection was confirmed in orchards with symptomatic samples. No infection by SLRSV was detected by ELISA test in the collected samples.

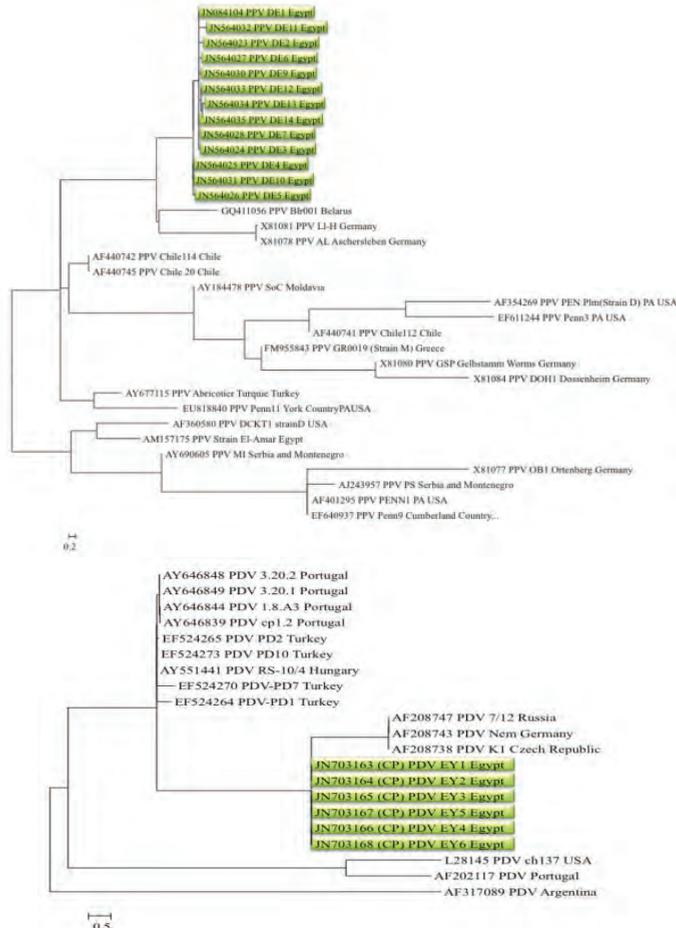


Fig.4 - Phylogenetic trees reconstructed from partial nucleotide sequences of **A**, CP gene and the 3' untranslated region of *Plum pox virus* (PPV), **B**, CP gene of *Prune dwarf virus* (PDV) isolates by the neighbor-joining method of MEGA4 (Tamura *et al.*, 2007). Bootstrap analysis was performed with 1000 replicates. The scale bar shows the number of substitutions per nucleotide.

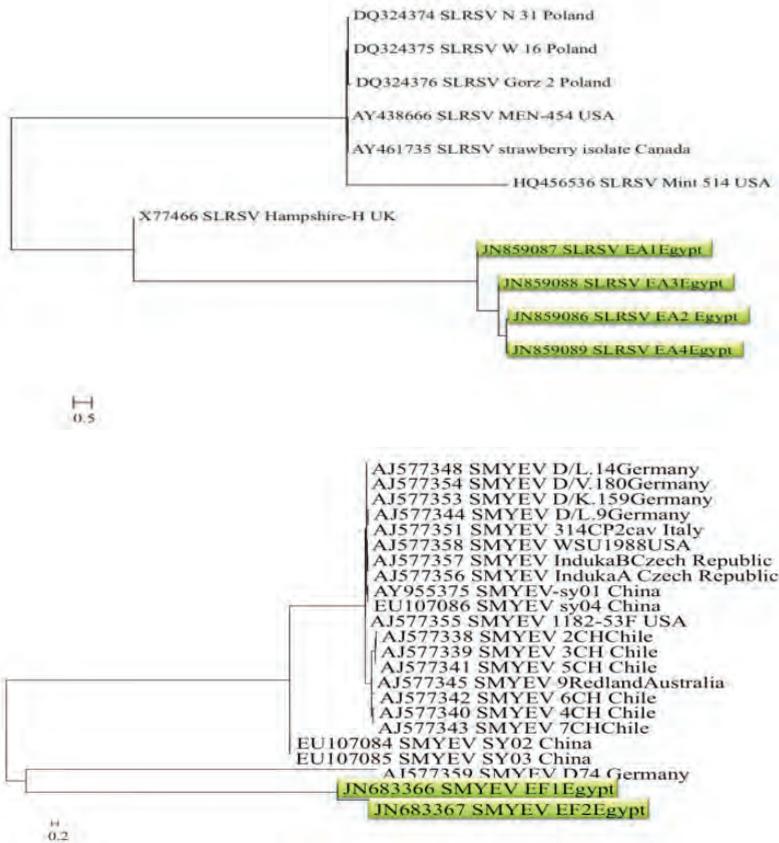


Fig. 5 - Phylogenetic trees reconstructed from partial nucleotide sequences of **A**, CP gene of *Strawberry latent ringspot virus* (SLRSV), and **B**, CP gene of *Strawberry mild yellow edge virus* (SMYEV) isolates by the neighbor-joining method of MEGA4 (Tamura *et al.*, 2007). Bootstrap analysis was performed with 1000 replicates. The scale bar shows the number of substitutions per nucleotide.

**RT-PCR analyses:** A total of 102 symptomatic samples of different species of stone fruits were processed by RT-PCR for the detection of stone fruit viruses (PPV, PDV, PNRSV, ACLSV and ApMV) and by RT-PCR followed by n-PCR for the detection of SLRSV. Results indicated that the overall infection in the surveyed Egyptian stone fruits by RT-PCR and n-PCR, was 27.4% (28 out of 102). 24.4% of the samples were infected with one virus and 3% were infected with more than one virus (mixed infection). PPV-Was the most widespread virus. It was detected in 19.6% (20/102) of all samples tested, followed by PDV, detected in 8.8% (9/102) of tested samples, followed by SLRSV detected in 3.9% (4/102) of tested samples. PNRSV, ACLSV and ApMV were not detected in any of the samples tested. PPV, PDV and SLRSV were found individually and in combinations. The most common findings were PPV alone

in 15.6%, followed by PDV in 3.9% and by SLRSV in 2.9% of the samples tested. Double infections of PPV and PDV viruses were also found in 3.9% of the samples, PDV and SLRSV in very few samples (0.98%) indicating low infection incidence. PPV was the most prevalent both in single and double infections. The highest incidence was recorded in El-Menofia (45%), followed by Alexandria (54%) and by El-Beheira (35%). The infection levels of different species was: 50% in plum (9/18), 16.6% in apricot (2/12) and 23.6% in peach (17/72). Regarding strawberry viruses, RT-PCR and n-PCR were used to confirm the presence of the target viruses in selected samples. Results indicated the absence of tested viruses in the surveyed area except SMYEV. The total average SMYEV infection was 20% (10/50) by RT-PCR followed by n-PCR, whereas it was 12% (6/50) by RT-PCR and just 6% (3/50) by DAS-ELISA. N-PCR proved to be a more sensitive tool for the detection of SMYEV in comparison with the ELISA and RT-PCR techniques. Also, results revealed that the highest incidence was recorded in reclaimed land (Bader centre) (23.3%), followed by recently reclaimed land (El-Nobarria) (15%). Festival and Sweet Charlie cultivars were the most susceptible to infection, followed by Rosa Linda and Camarosa with an incidence of 28%, 26.6%, 12.5% and 11%, respectively.

**Partial sequencing of the Egyptian isolates clones of viruses:** DNA products of the expected size were obtained by RT-PCR from total RNA extracted from leaves infected with PPV (593-bp fragment of the CP coding region), PDV (204 bp) and SLRSV (203 bp). No viral DNA amplicon was obtained from total RNA from leaves of healthy samples. DNA amplicons obtained by RT-PCR for all isolates of PPV, PDV and SLRSV were cloned and sequenced. Nucleic acid sequences (13 for PPV, 6 for PDV and 4 for SLRSV) determined in this study were made available in GenBank as accession numbers JN655521 to JN655523 for PPV, JN703163 to JN703168 for PDV and JN859086 to JN859089 for SLRSV. Sequence analysis confirmed the respective viral nature of RT-PCR products. Multiple sequence alignments indicated that PPV, PDV and SLRSV isolates from Egypt and 21 PPV, 15 PDV, and 7 SLRSV isolates from other geographic origins had 92 to 99%, 84% to 93%, and 7% to 73% identity at the nucleotide level, respectively. DNA products of the expected size were obtained from total RNA from strawberry leaves infected with SMYEV (292-bp fragment of the CP coding region). No viral DNA amplicon was obtained from total RNA from leaves of healthy samples. DNA amplicons obtained by RT-PCR for all isolates of SMYEV each were cloned and sequenced. SMYEV nucleic acid sequences determined in this study were made available in GenBank as accession numbers JN683366 to JN683367. Sequence analysis confirmed the respective viral nature of RT-PCR products. Multiple sequence alignments indicated that two SMYEV isolates from Egypt and 21 SMYEV isolates from other geographic origins had 81 to 98% identity at the nucleotide level.

**Comparison of partial nucleotide sequences of the Egyptian isolates of viruses with other reported isolates:** Phylogenetic relationships among viral isolates were inferred using MEGA version 4, (Bootstrap test of phylogeny, Neighbor-Joining) for a 593-nt portion of the PPV CP gene and the 3' untranslated region, for a 836-bp fragment of the PPV P3-6K1 genomic region, for a 204-nt fragment of the PDV CP gene and for a 203-nt segment of the SLRSV CP gene. Regarding PPV, all Egyptian acces-

sions showed high phylogenetic distance against the recombinant isolate 'Serbia-MI' from Serbia and Montenegro (92% to 93% of sequence identity), against PPV-Marcus isolates (93% to 95%) from Greece and from Serbia and Montenegro, against the sour cherry isolate (PPV-C) from Moldavia (93% to 94%) and against PPV-El Amar isolate (92% to 93%). A higher sequence identity was detected against one PPV-D East-European isolate from Belarus (98% to 99%) and against PPV-D isolates from USA (98.0% to 99%), Chile (97% to 99%) and Germany (97% to 99%) confirming that all PPV Egyptian isolates investigated were classified as Dideron strain. Phylogenetic analyses revealed that 13 Egyptian PPV isolates from apricot, peaches and plum grouped together in the same branch and appeared to be closely related. The next closest related D isolate was Blr001 from Belarus (GQ411056) as shown in Fig. (4A). Regarding PDV, all Egyptian accessions showed a higher phylogenetic distance against the PDV almond isolates from Portugal (84% to 87% of sequence identity), against PDV cherry isolate (87%) from Hungary and against PDV cherry isolates (81% to 84%) from Turkey. However, the sequence identity was detected against PDV peach isolates from Germany (93%), against PDV plum isolate from Russia (93%), against PDV cherry isolate from Czech Republic (93%) and against PDV isolate (93%) from USA as shown in Fig. (4B). Phylogenetic analyses revealed that our 6 Egyptian isolates from peach (JN703163, JN703164, JN703165 and JN703166) and from plum (JN703167 and JN703168), grouped together in the same branch. The next-closest isolates were from Czech Republic (AF208738), Germany (AF208743) and Russia (AF208747). Regarding SLRSV, all Egyptian accessions showed higher phylogenetic distance against the Polish black locust isolates SLRSV-G2 (7% to 9%), SLRSV-W16 and N31 (6% to 9%), against the strawberry isolate from Canada (7% to 8%), against the mint isolate from USA and against the strawberry isolate (SLRSV-H) from the United Kingdom (70% to 73%). All Egyptian peach isolates of SLRSV (JN859086 to JN859089) were distinct from all other SLRSV isolates as shown in Fig. (5A). It should be noted that geographical location-associated clustering of Egyptian PPV, PDV and SLRSV isolates was observed. Phylogenetic relationships among viral isolates were inferred using MEGA version 4, (Bootstrap test of phylogeny, Neighbor-Joining) for a 292-nt portion of the SMYEV CP gene. Although, the nucleotide distance for the 2 Egyptian isolates EF1, EF2 indicated 88%, 2 Egyptian SMYEV isolates were clustered in the same branch. The next-closest isolate was D74 isolate from Germany (AJ577359) as shown in Fig.(5B). It should be noted that geographical distribution-associated clustering was observed for the Egyptian SMYEV isolates as well as most of the previously reported SMYEV isolates.

## Discussion

The Mediterranean region produces about 40% of the world's supply of stone fruits (Myrta *et al.*, 2003). Stone fruit trees in Egypt are very important as they account for 6% of the total fruit production. Our study addressed the most important plant viruses known to infect the most important stone fruit crops in Egypt namely apricot, peach and Japanese plum. Detection of such viruses was attempted by three different diagnostic tools: symptomatology, ELISA and molecular techniques. Survey and sample collection were carried out in three different governorates (El-Beheira, Alexandria and El-Menofia) representing reclaimed, recently reclaimed and old lands. Our survey showed that PPV-Was the only virus with characteristic symptoms on apricot, peach and plum in old lands. Distribution of symptoms was scattered in surveyed areas. However, PDV symptoms were detected only on peach trees. Symptoms were also irregularly scattered and no leaf symptoms observed. Plants exhibited stunting, rosette formation and reduction of plant growth and fruit production. As for PNRSV, ACLSV, ApMV and SLRSV, no characteristic symptoms were detected in any of the surveyed orchards and nurseries.

PPV and PDV visual symptoms were very mild during the winter survey. The expression of symptoms is influenced by several factors like variety, age of the host, virus titer and period of the year. It is reported that PPV shows an uneven distribution in infected trees and its titer decreases during the summer and winter periods (Capote *et al.*, 2009). It is reported that hot climate and iron deficiencies conditions probably masked the symptoms (El Maghraby *et al.*, 2007). Our survey was conducted in winter and it is our observation that symptoms were masked by cold weather conditions (PPV titer was low). Previous reports on viruses of stone fruits in Egypt indicate an absence of symptoms in visited orchards encompassing a wider area of recently reclaimed and old lands of seven governorates including those under study (El Maghraby *et al.*, 2007). The exception was for Sharka disease symptoms on local apricot cultivar in old lands (Mazyad *et al.*, 1992). In agreement with other reports (Mazyad *et al.*, 1992; Aboul-Ela and Amal, 1994; Aboul-Ela *et al.*, 1999; Shalaby *et al.*, 2003; El Maghraby *et al.*, 2007), no PPV-Were detected in new reclaimed lands in the El-Beheira governorate (El-Nobarria). PPV-Were found only in old lands of El-Menofia, El-Beheira and Alexandria governorates. In the sampled fields in Alexandria, no PPV symptoms were seen although, in agreement with another report (Shalaby *et al.*, 2003), its presence was reported. PDV symptoms were however more obvious on peach in Alexandria.

Although the distribution of aphids was high in most of the surveyed areas, this was not proportional to symptom distribution suggesting a low natural spread of the virus by aphids. It is reported that aphids are not very efficient in transmitting the D strain of PPV-Which is referred to as the non-epidemic form of PPV (Levy *et al.*, 2000). It should be mentioned that our molecular study classifies the identified PPV isolates in this study as belonging to the D strain. The search for more sensitive diagnostic techniques than ELISA involved developing sensitive RT-PCR assays for PPV and PDV to be used in basic research and by quarantine and certification agencies for viral detection and control (Hadidi and Candresse, 2001). By applying RT-PCR using virus-specific primers (designed on conserved regions of each virus), we detected

PPV, PDV and SLRSV. No infection by PNRSV, ACLSV and ApMV was detected by RT-PCR. The higher degree of sensitivity of RT-PCR was clear in the incidence of PPV and PDV in the collected samples. An incidence in peach of 12.5% for PPV and 9.72% for PDV by RT-PCR compared to 7% and 5.6% by ELISA was reported. However, incidence was identical for apricot and plum samples for PPV. RT-PCR followed by n-PCR was also more sensitive in the detection of SLRSV in peach (5.5% compared to 0.98% by ELISA).

PPV-Was recorded only in old lands, where it was detected before with a higher incidence (Aboul-Ela *et al.*, 1999). This may be due to the replacement of many stone fruit orchards in old lands with new crops e.g. citrus and mango since the late 1990s. PDV incidence was low therefore we suggest that PDV infection is spreading slowly; it is conceivable that the propagation of materials by grafting reflects the uneven distribution of PDV in the plant materials that were introduced. It is reported that PDV has been found only in low incidence in Egypt (El Maghraby *et al.*, 2007). SLRSV incidence was also low. Virus infection is spreading slowly perhaps because SLRSV is transmitted by the soil nematode *Xiphinema diversicaudatum* and the transmission from plants to plants is slow, reflecting the restricted mobility of the nematode. Also, it is reported that SLRSV is generally less frequent on *Prunus* sp. in the Mediterranean region (Myrta *et al.*, 2003). The fair sanitary status of newly reclaimed lands and the poor sanitary status of old cultivated areas of the fruit tree industry, as appears from the present work, suggests that the implementation of a national certification program could be highly beneficial for a rapid improvement of the local nursery and fruit industry and would successfully limit the dissemination of stone fruit viruses.

*Plum pox virus* (PPV) is the most devastating viral disease worldwide of stone fruit. There are several known strains of the virus PPV-M, D, C, Rec, EA and W strains (Glasa and Candresse, 2008). The detection and identification of its strains are therefore of critical importance to plant quarantine and certification programs. In order to identify the PPV strain present in Egypt, a primer pair (Sprimer/M4T) which detects portion of the coat protein and the complete 3' untranslated region in the 3' terminus of the genome was used for amplification and cloning of about 1700 nucleotides of 20 samples. Sequencing of RT-PCR amplified DNA fragments confirmed the identity of the virus and indicated the occurrence of different Egyptian PPV isolates. CP partial sequence of 13 Egyptian PPV isolates was determined and all sequences deposited in the GenBank. All Egyptian accessions showed higher sequence identity against PPV-D isolates. This is the first report of the PPV-D isolate in Egypt. All previous studies reported the presence of the PPV-EI Amar since the 1980s (Wetzel *et al.*, 1991; Mazyad *et al.*, 1992; Aboul-Ela and Amal, 1994; Ghanem *et al.*, 1997; Abdel-Ghaffar *et al.*, 1998; Aboul-Ela *et al.*, 1999; Myrta *et al.*, 2003; Shalaby *et al.*, 2003; Salama *et al.*, 2003; Myrta *et al.*, 2006).

The detection of the PPV-D in Egypt in all 13 cloned isolates confirms its establishment in a new geographic area. The best explanation for this is that international trade of agricultural products and parts of plants, has broken the traditional barriers to the movement of pathogens. As exchange of plant parts for propagation, for example, has become more international, the potential for the importation of nonindigenous plant viruses has increased significantly. This is very clear from the absence of the

detection of PPV-EI Amar isolates, known to be indigenous in Egypt, and the appearance of the PPV-D isolate. This may simply be due to the termination or decline of the use of some local cultivars and importation of other cultivars with different or better characteristics. This strongly illustrates the necessity of enforcement of plant exchange certification programs that must be vigorously implemented in Egypt. Our results show a high incidence of PPV-D infected plants in orchards and nurseries in the Alexandria governorate. The presence of the Dideron strain, considered the least epidemic strain of PPV-Due its low efficiency in aphid-transmission (less widespread in the field), suggests a large diffusion of PPV-infected material used for plant propagation. For this reason an implementation of the certification schemes is necessary in Egypt in order to guarantee the production and the employment of virus-free propagating material.

The partial sequencing of the coat protein gene of 8 Egyptian PDV isolates was determined; all sequences were deposited in the GenBank and compared with those of other PDV variants to reveal and understand the variability which may help in controlling this virus. All Egyptian accessions (from peach and plum) showed a close relation with peach isolates from Germany, Czech Republic and with plum isolates from Russia. This is in agreement with the report by Youssef *et al.* (2002). Our study did not find host-based clustering, the clustering of Egyptian PDV isolates from peach and plum samples in the same group was observed may be due to transmission of the virus by grafting. This is in agreement with earlier reports (Fonseca *et al.*, 2005; Ulubas *et al.*, 2009). However, the geographical location-associated clustering of Egyptian PDV isolates from peach and plum samples was observed in our study, in contrast to the earlier reports to this effect of PDV (Fonseca *et al.*, 2005; Ulubas *et al.*, 2009). This could be explained by the simple transmission of PDV by vegetative material during intensive exchanges of biological propagation material in the past. Our results show the incidence of PDV infected plants in orchards in the selected governorates of Egypt suggesting the diffusion of PDV-infected material used for plant propagation. For this reason the implementation of the certification schemes is necessary in Egypt in order to guarantee the production and the employment of virus-free propagating material. Partial sequences of four Egyptian SLRSV isolates were determined and all sequences were deposited in the GenBank. All Egyptian accessions showed a higher phylogenetic distance from all other SLRSV isolates. Our results showed that the four Egyptian SLRSV isolates were highly distinct from other mentioned SLRSV isolates available in GenBank. It showed a low sequence identity at the nucleotide level with the other isolates therefore further studies are necessary to understand whether the Egyptian SLRSV isolates (JN859086-JN859089) may represent a new strain of SLRSV.

A survey of strawberry (*Fragaria x ananassa*) commercial fields and nurseries in El- Beheira governorate in 2009/2010 was conducted for the collection of leaf samples showing virus-related symptoms. Major symptoms of leaf rolling, yellowing, marginal chlorosis, purple discoloration, stunting, decline, reduction in size and distortion or mottling indicative of viral infection were collected (Ratti *et al.*, 2008; Dougdoug *et al.*, 2010). The observation that infected plants were usually found in confined patches within the field may be explained by the mode of transmission of the viruses by aphid, white flies, oomycetes or nematode vectors (Martin and Tzanetakis,

2006). This is in agreement with previous report (Ratti *et al.*, 2008). It is a common understanding that the detection of strawberry viruses by PCR is not easy depending on the quality of the extracted nucleic acid as the presence of secondary metabolites (rich in a range of phenolics and polysaccharides) were shown to have direct inhibitory effects (Thompson *et al.*, 2003). Total nucleic acid extraction by the modified CTAB method (Chang *et al.*, 2007), and total RNA extraction by the modified CTAB method (Chang *et al.*, 1993) as well as the use of a commercially available plant RNeasy kit (GE Healthcare, UK) were compared as template for the detection of the strawberry viruses. Some reliable methods for the extraction of high quality total RNA from strawberry plants have been reported (Thompson *et al.*, 2003; Yang *et al.*, 2005), but they have limitations, for example, the RNA isolation kit is expensive and the modified CTAB method is time-consuming. Extraction of total nucleic acid with a modified CTAB method is easy, fast and reasonably economical (Chang *et al.*, 2007). No significant difference between different nucleic acid extraction method were detected in our study. However, because the strawberry viruses tested include DNA (SVBV) and RNA (SCV, SMYEV, SLRSV and SMoV) viruses, the extraction of total nucleic acid from strawberry plant with the CTAB method may be preferable as it allows the easy detection of both RNA and DNA viruses by RT-PCR. This makes an investigation of the epidemiology of strawberry viruses much easier. All strawberry viruses tested by RT-PCR were absent in the collected samples except SMYEV.

The use of n-PCR was observed as the most sensitive method for the detection of SMYEV. Comparing the sensitivity of SMYEV detection by different diagnostic methods indicates an efficiency of 6% by ELISA, 12% by RT-PCR and 20% by n-PCR. Phylogenetic analysis of the Egyptian SMYEV isolates indicated that they occupy a separate cluster from other isolates from different geographic regions. However, similarity with a D74 isolate from Germany (AJ577359), suggests a diffusion of SMYEV-infected material used for plant propagation. For this reason the implementation of certification schemes is necessary in Egypt in order to guarantee the production and the employment of virus-free propagating material. Phylogenetic analysis of the SMYEV isolates indicated a correlation between geographic region and strain variability. This is in agreement with earlier reports (Li and Yang, 2009, 2011). Results emphasize the importance of certification programs to restrict virus introduction through the uncontrolled exchange of plant material by the use of the more sensitive, optimized molecular detection methods.

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***RASPBERRY LEAF BLOTCH VIRUS, A NEW EMARAVIRUS  
INFECTING RASPBERRY***

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In the last 3 or 4 years raspberry leaf and bud mite (*Phyllocoptes gracilis*; RLBM) has emerged as a serious cause of disease in some raspberry plantations in England and Scotland. Chemical treatments to control the mite are either being removed from use by EU directive or are ineffectual, and the incidence of the mite is increasing. We have recently identified a virus, *Raspberry leaf blotch virus* (RLBV), which is transmitted by the mite and could be a significant contributing factor to the disease severity. Sequencing of RLBV revealed that it has a genome comprised of five RNAs of negative strand polarity, with the complementary strand of each RNA molecule potentially expressing a single protein. This genome structure and viral protein sequences suggest that RLBV is a new member of the very recently described *Emaravirus* genus of plant viruses. Other members of this genus include, *European mountain ash ringspot associated virus*, *Fig mosaic virus* and *Rose rosette virus*, all of which are transmitted by mites.

## EMERGING DISEASE IN BLUEBERRY CAUSED BY A NOVEL RNA VIRUS

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A new disorder was observed on southern highbush blueberry (*Vaccinium corymbosum* interspecific hybrids) in several southeastern states. Symptoms included irregularly shaped circular spots or blotches with green centers on the top and bottom of leaves. The disease was reported initially in the state of Georgia in 2006 and 2007; but in 2008, it was found in production areas in the states of Florida, Mississippi, South Carolina, and North Carolina. Diagnostic tests failed to isolate any fungal or bacterial pathogens typically associated with such symptoms. Double-stranded RNA (dsRNA) extracted from symptomatic leaves suggested the presence of virus(es) possibly involved in the disease. Sequencing revealed the presence of a novel virus with a genome of ~14 kb divided into 4 positive-sense RNA segments. Primers developed from conserved areas of the RdRp detected the virus in more than 50 individual plants that exhibited necrotic ring blotch symptoms in North Carolina, Georgia and Florida. The perfect correlation between the virus and symptoms in plants from across several states suggests that the virus, for which we propose the name *Blueberry necrotic ring blotch virus* (BNRBV), is the causal agent of the disease. Sequence analysis showed that BNRBV possesses protein domains conserved across ssRNA viruses from the *Virgaviridae*, *Bromoviridae* and the newly created genus *Cilevirus*. Furthermore, BNRBV has two Helicase (HEL) domains located on RNA 1 and 2. Both HEL domains possess conserved motifs and, more importantly, represent two distinct clades indicating virus recombination events as a result of co-infections.

## OCCURRENCE AND VARIABILITY IN EUROPEAN ISOLATES OF *BLUEBERRY RED RINGSPOT VIRUS*

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The red ringspot disease of highbush blueberry (*Vaccinium corymbosum* L.) identified in the USA by Hutchinson and Varney (1954) is caused by *Blueberry red ringspot virus* (BRRV) genus *Soymovirus*, family *Caulimoviridae*, with circular ds-DNA genome and particles of 42 – 46 nm in diameter (Kim *et al.*, 1981). Complete genome sequence of the New Jersey (NJ, USA) isolate was described by Glasheen *et al.*, (2002). Recently, BRRV was detected in Japan (Isogai *et al.*, 2009), Slovenia (Pleško *et al.*, 2009) and Poland (Kalinowska *et al.*, 2012). In the Czech Republic BRRV was found after extensive survey in 2 plants of cv. Darrow with reddish ring spots and blotches on the upper surface of leaves in a field germplasm collection in South Bohemia in 2009 (Příbylová *et al.*, 2010). The electronmicroscopical examination of ultrathin sections prepared from leaves showed spherical virus particles present in inclusion bodies formed by an electron-dense matrix with electron-lucent area enclosed in an endoplasmic reticulum. Complete genomes of Czech and Slovenian isolates of BRRV were sequenced. Their comparisons showed 95.9% identity of both isolates but only 92.2% identity with the US NJ isolate. Phylogenetic analysis performed on a 186-aa-long region from the reverse transcriptase protein of 17 known isolates identified four clusters: one containing Japanese sequences from cvs. Weymouth and Blueray, one branch of a single American isolate from cranberry (Cran I), cluster containing cvs. Sierra and Duke isolates from Japan and less defined cluster containing the other US isolates together with the two European ones. We conclude that the phylogenetic tree more probably reflects natural variability of American BRRV isolates present in imported rootstocks rather than variability generated in the field. Experiments on BRRV elimination from cv. Darrow are in progress.

### Acknowledgements

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## **DISSECTING THE EPIDEMIOLOGY OF *BLACKBERRY YELLOW VEIN ASSOCIATED VIRUS* AND *BLACKBERRY CHLOROTIC RINGSPOT VIRUS*; A STUDY ON POPULATION STRUCTURE, TRANSMISSION, AND ALTERNATIVE HOSTS**

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### **Abstract**

Blackberry yellow vein disease (BYVD), a disorder caused by virus complexes, is the most important blackberry disease in the southeastern United States. *Blackberry yellow vein associated virus* (BYVaV) is the most prevalent virus in BYVD plants. *Blackberry chlorotic ringspot virus* (BCRV) is another virus frequently found in BYVD plants, and in addition to blackberry, it can also infect rose and raspberry.



Fig. 1 - Different symptoms associated with Blackberry yellow vein disease. Left: vein banding and right: irregular chlorosis.

Despite the importance of the viruses as major components of BYVD little is known about their epidemiology. In this study population diversity, transmission avenues and potential alternative hosts of both viruses were studied. The variability among BYVaV isolates was determined after sequencing four genomic regions of 34 isolates. The diversity of BCRV was assessed by obtaining the complete sequence of RNA

3 of 30 isolates from three different hosts. Whiteflies were tested for their ability to transmit BYVaV and seed transmission of BCRV was evaluated using two hosts. Twenty-five species of plants were tested as potential alternative hosts of the viruses.

### Introduction

Recent increases in acreage and expansion to new areas has led to the emergence of new blackberry disorders with blackberry yellow vein disease (BYVD) becoming a major problem in the southeastern United States. Typical symptoms include vein yellowing of primocane leaves, with new leaves usually being asymptomatic (Susaimuthu *et al.*, 2007). Symptoms also include oak-leaf patterns, irregular chlorosis and line patterns (Susaimuthu *et al.*, 2006) (Fig. 1).

Floricanes can be severely affected by the disease, resulting in dieback of canes during the fruiting season. BYVD was initially mistaken for *Tobacco ringspot virus* (TRSV) infection; however, grafting experiments proved that TRSV is asymptomatic in many blackberry cultivars (R. Gergerich, unpublished). Further studies were carried out to determine the causal agent(s) of the disease. Martin *et al.* (2004) reported a new crinivirus in symptomatic samples named *Blackberry yellow vein associated virus* (BYVaV). Although all symptomatic plants in the Martin *et al.* study were infected with BYVaV, further screening indicated that BYVaV is latent in single infections (Susaimuthu *et al.*, 2008a). It was thus speculated that BYVaV synergizes with other viruses to cause disease. Subsequently, several other viruses were isolated from symptomatic plants exhibiting BYVD, including *Blackberry chlorotic ringspot virus* (BCRV) (Tzanetakis, 2007b) and several others (Susaimuthu *et al.*, 2008b; Tzanetakis *et al.*, 2009; Sabanadzovic and Abou Ghanem-Sabanadzovic, 2009; Sabanadzovic *et al.*, 2011). BCRV is a subgroup-I ilarvirus first isolated from blackberry in the UK (Jones *et al.*, 2006) and rose in the United States (Tzanetakis, 2006a). The virus has also been found to naturally infect raspberry. As BCRV infects three rosaceous hosts, there is always the possibility of additional hosts in the family being infected with the virus.

### Materials and Methods

Fully-matured young leaves were collected from commercial fields as well as wild blackberry, showing BYVD symptoms. Samples were collected from blackberry in Arkansas, California, Missouri, Georgia, Mississippi, Oregon, North Carolina, and South Carolina. BCRV samples were also collected from rose and raspberry.

**RT-PCR:** Total nucleic acid extractions were performed as described (Tzanetakis *et al.*, 2007a). Purified nucleic acids were used in reverse transcription (RT). Multiple sets of detection primers were used for each virus to detect the highest possible number of virus isolates. The samples were visualized in a 2% TBE- agarose gel stained with GelRed® (Biotium Inc.).

**Virus diversity:** Samples that tested positive by RT-PCR for BYVaV and BCRV were used in the diversity study. Sequence information for a total of 34 and 30 isolates was obtained for BYVaV and BCRV respectively (Tab. 1). For the diversity study of BYVaV four different regions of the virus genome were amplified and cloned. Primers were designed to amplify the coding region corresponding to the polyprotein (region 1); polymerase (region 2); p4 and part of HSP70h (region 3); and part of the coat protein and minor coat protein (region 4). A total of 4512 nucleotides were analyzed per isolate which represents almost 30% of the virus genome. RNA 3 is the least conserved genomic fragment of all members of *Bromoviridae* (Tzanetakis *et al.*, 2010). For this reason RNA 3 was chosen for diversity study on BCRV, representing 28% of the virus genome. Primers were designed at the 3' and 5' end of the RNA to obtain the sequence of complete RNA 3 of the virus. The PCR products were purified and cloned using the TOPO-TA cloning Kit (Invitrogen). At least three clones were sequenced for each of the BYVaV and BCRV isolates.

**Sequence analysis:** Sequences were analyzed with CAP3 (Huang and Madan, 1999) to obtain sequence contigs. Diversity among isolates was determined with Bioedit (Hall, 1999). Phylogenetic and molecular evolutionary analyses were conducted on MEGA v.5 (Tamura *et al.*, 2011). The phylogenetic trees were generated on MEGA v. 5, implementing the Maximum likelihood method with 1000 bootstrap replicates and the Kimura 2-parameter model (Kimura, 1980).

**Virus transmission:** Transmission tests for BYVaV were performed at the USDA-ARS, Salinas, CA. The greenhouse (*Trialeurodes vaporariorum*) and banded-winged (*Trialeurodes abutilonea*) whiteflies were tested for their ability to transmit the virus. Large numbers of each whitefly species were allowed acquisition access periods (AAPs) of 18 to 24 hours whitefly on blackberry plants (variety Natchez), singly infected with BYVaV. Following virus acquisition, whiteflies were transferred to leaf clip-cages (50 whiteflies per cage) and attached to the underside of virus-free blackberry leaves (variety Chester Thornless) for an additional 48 hours. Seed-transmissibility of BCRV was evaluated on wild rose naturally infected with BCRV. The virus was also mechanically transmitted to *Chenopodium quinoa* and seed-transmission rate was evaluated on this host. For each plant species, 100 seedlings were tested to determine transmission efficiency. Infection was confirmed by nucleic acid extraction and RT-PCR as described above.

**Alternative host testing:** Alternative hosts can play an important role in virus epidemiology by acting as reservoirs for a virus. For this reason, we tested 25 different plant species as potential alternative hosts (Tab. 2). Plant species were collected from areas with high BYVaV and BCRV incidence with at least 16 plants/species tested by RT-PCR.

## Results

When BYVaV isolates were aligned, region 1 had an average nt identity of 94%. For regions 2, 3 and 4 the average nt identity was 95-96%. BCRV isolates were very similar, which was somewhat surprising considering that they were collected from different states, hosts, and wild vs cultivated plants. The nucleotide identity between U.S. isolates of BCRV ranged from 95 to 100%; however, all US isolates showed less than 90% nt identity with the UK blackberry isolate (GenBank accession DQ091195), the only other BCRV sequence found in GenBank at the time of completion of this study. The CP coding region of RNA 3 of BCRV was more diverse compared to the MP counterpart or the complete RNA 3 sequence. Phylogenetic analysis of BYVaV isolates did not give any discernable pattern of geographical isolation or similarity within in-state populations. As U.S. BCRV virus populations were homogenous no distinct geographic or host-specific patterns were observed.

**Transmission:** Two whitefly species, *T. vaporariorum* and *T. abutilonea* were able to transmit BYVaV with high efficiency. BCRV was seed-transmissible at rates close to 50% for *C. quinoa* and *Rosa multiflora*.

**Alternative hosts:** BYVaV was not detected in any species tested as potential alternative hosts. BCRV was detected in two apple samples. The samples tested positive in two consecutive PCRs and the products were sequenced to confirm the presence of the virus.

## Discussion

Studies on the epidemiology of two important viruses of the BYVD complexes is presented; focusing on three aspects of virus epidemiology: diversity, transmission and alternative hosts.

Thirty-four BYVaV isolates collected from five states were examined. Phylogenetic analysis did not show any particular pattern as isolates showed inconsistent clustering. This indicated possible recombination within the virus population, an important evolutionary trait in the family *Closteroviridae* (Karasev, 2000). Given the diversity of the blackberry isolates it appears that the initial sources of infection are wild blackberries, an idea reinforced with the identification of two whitefly species as vectors of the virus. This is an important issue given that the identified whitefly species have a relatively wide host range (Wintermantel, 2004). Results will help growers to reduce BYVD associated losses by insect monitoring in the field.

There might be several reasons for not identifying any other-than-blackberry natural infections of BYVaV in the field. It has been reported that BYVaV titers cycle in host plants during mixed infections which may cause the virus to go undetected (Susaimuthu *et al.*, 2008a). Additionally, criniviruses generally have a narrow host range. This appears to be the case for BYVaV as the virus was unable to infect any of tested non-blackberry plants.

For BCRV, we studied 30 isolates collected from five states and three hosts; blackberry, raspberry and rose. In our study virus sequences showed that U.S. isolates

are closely related despite different hosts assayed, with all isolates being different when compared to the UK counterpart. The mode of transmission of the virus, the propagation practices and exchange of germplasm may play a role in the lack of clustering based on host species or geographical origin. The higher seed transmission rates observed should be of concern to plant breeders. Plants infected with virus from the seed can serve as a potential source of infection in the field. The fact that BCRV was found naturally infecting apple, is of great concern as there might be more species from this taxon infected by the virus.

Tab. 1. Isolates used in the diversity study of Blackberry yellow vein associated virus and Blackberry chlorotic ringspot virus

State	Plant species	Wild/total isolates
<b>BYVaV</b>		
Arkansas	Blackberry	6/13
Georgia	Blackberry	0/3
Mississippi	Blackberry	0/3
North Carolina	Blackberry	0/7
South Carolina	Blackberry	0/8
<b>BCRV</b>		
Arkansas	Rose	10/13
Arkansas	Blackberry	10/12
California	Blackberry	0/1
Missouri	Rose	2/2
Oregon	Raspberry	0/2

Tab. 2 - List of plant species tested as potential alternative host of *Blackberry yellow vein associated virus* and *Blackberry chlorotic ringspot virus*.

Plant species	Scientific name	Family	plants
Garden vetch	<i>Vicia sativa</i>	Leguminosae	16
Virginia creeper	<i>Parthenocissus quinquefolia</i>	Vitaceae	16
Red Clover	<i>Trifolium pretense</i>	Fabaceae	16
Wild garlic	<i>Allium vineale</i>	Amaryllideaceae	16
Creeping woodsorrel	<i>Oxalis corniculata</i>	Oxalidaceae	16
Carolina geranium	<i>Geranium carolinium</i>	Geraniaceae	16
Curly dock	<i>Rumex crispus</i>	Polygoneaceae	16
Dandelion	<i>Taraxacum officinale</i>	Compositae	16
Tall fescue	<i>Fetuca arundinaceae</i>	Poaceae	16
Wild wheat	<i>Avena fatua</i>	Poaceae	16
Grapes	<i>Vitis vinifera</i>	Vitaceae	16
Peach	<i>Prunus persica</i>	Rosaceae	16
Blueberry	<i>Vaccinium spp.</i>	Ericaceae	16
Shepherds purse	<i>Capsella bursa pastoris</i>	Cruciferaeae	16
Nutsedge	<i>Cyperuss Spp.</i>	Cyperaceae	16
Horsenettle	<i>Solanum Carolinense</i>	Solaneceae	16
Common ragweed	<i>Ambrosia artemisifolia</i>	Asteraceae	16
Tree of heaven	<i>Alianthus altissima</i>	Simaroubaceae	16
Apple	<i>Malus spp.</i>	Rosaceae	185
Rose	<i>Rosa multiflora</i>	Rosaceae	40
Carpetweed	<i>Mogullo verticillate</i>	Molluginaceae	16
Amaranthus	<i>Amaranthus spp.</i>	Amaranthaceae	16
Poor joe	<i>Diodia teres</i>	Rubiaceae	16
Ground Cherry	<i>Physalis spp.</i>	Solanaceae	16
Sorghum	<i>Sorghum spp.</i>	Poaceae	16

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## **PHYTOPLASMAS OF GROUP 16SrI ASSOCIATED WITH STRAWBERRY (*FRAGARIA X ANANASSA*) IN COLOMBIA**

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Cajicá (15 Km north of Bogotá-Colombia) is a small town located at 2,600 m altitude on the Andes cordillera. In June 2010, an experimental semi-organic strawberry (*Fragaria X Ananassa*) crop was planted under a plastic greenhouse to test fruit yield of the 'Ventana' variety. In 2011, the crop was examined in order to determine the cause of a dramatic drop in production. Symptoms such as fruit phyllody, flower virescence, leaf reddening, abnormal shoot production and changes in sepal size were observed. Leaves from 4 symptomatic plants were sampled and DNA extracted by a CTAB extraction method, followed by an additional cleaning step (Power Clean DNA MoBio). Nested PCR tests were performed with P1A/P7A-R16F2n/R16R2. Amplicons were purified and later sequenced using R16F2n/R16R2 primers, but *Bacillus* spp. was detected. However, when fU3/rU5 primers were used, a band of 845 pb was obtained and phytoplasma sequences were found. The sequence was compared with NCBI database using BLAST and a homology of more than 99% with phytoplasmas of group 16SrI was found. A dendrogram obtained using previously reported sequences confirmed the relationship of phytoplasmas with the aster yellows group ('*Candidatus* Phytoplasma asteris'-related strains). Although symptoms indicating the presence of phytoplasmas were observed in all tested samples, the presence of this pathogen was confirmed only in one of the evaluated strawberries plants. This is the first report of phytoplasma presence in strawberry in Colombia.

## **DETECTION OF VIRUSES AFFECTING *SAMBUCUS* SPP. PLANTS IN POLAND**

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*Sambucus* spp. plants are cultivated in Poland for fruit production and as ornamental shrubs. Fruits of elderberries are used for preserves and for wine-making, and many different elderberry parts are used for the production of pharmaceutical components. Studies conducted in the U.S. and Western Europe indicated that the greatest losses in the elderberry production are caused by viruses. Some of these viruses may cause in stone fruit cultivars. In 2009-2010 virus-like symptoms including chlorosis, rings or line patterns on the leaves and retarded growth of the whole plants were observed on *Sambucus nigra* and *S. racemosa* grown in commercial plantations as well as natural habitats. Samples from symptom-showing leaves were tested for the presence of 11 viruses using biological test, ELISA or RT-PCR. Out of 46 plants tested, 17 were infected with *Cherry leaf roll virus* (CLRV), one with *Arabis mosaic virus* (ArMV), two with *Tomato black ring virus* (TBRV) and four with *Elderberry latent virus* (EILV). Mixed infection of two viruses: TBRV and CLRV or TBRV and ArMV were found in two wild elderberry plants whereas commercial *S. nigra* cv. Aurea and *S. racemosa* cv. Sutherland Gold were infected with CLRV and EILV. To our knowledge, this presentation is the first report on EILV in *S. nigra* and *S. racemosa* plants.

## EPIDEMIOLOGICAL STUDIES ON LETHAL REDNESS DISEASE OCCURRING IN STRAWBERRY CROPS IN ARGENTINA

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In strawberry (*Fragaria x ananassa* D. cv camarosa) plants, the lethal redness symptoms are characterized by stunting, curled leaves and redness on the abaxial face, followed by a generalized decline, necrosis and subsequent death of the plant. These symptoms cause yield losses by preventing proper fruit development. Previous work reported the association of those symptoms with at least two different phytoplasmas. The strawberry read leaf phytoplasma (16SrXIII, subgroup E) and the strawberry X-disease phytoplasma (16SrIII) which have been exclusively associated with strawberry in Argentina. The aim of this work is to study the relationship between these and other phytoplasmas, with the symptoms described and to determine the incidence and prevalence of the described symptom and the percentage of diseased plants, in strawberry crops in Argentina. During the period 2010-2011 40 plots of strawberry fields located in Lules (Tucumán province, northwest Argentina), the most important production region in Argentina, were sampled. Incidence ( $I = \frac{N^{\circ} \text{ of plants with symptoms}}{N^{\circ} \text{ total of plant counted}}$ ), prevalence ( $P = \frac{\text{presence of at least 1 plant with symptoms per plot}}{N^{\circ} \text{ total of plots analyzed}}$ ) and percentage of infected plants ( $\frac{\text{No. of PCR-positive samples for phytoplasmas}}{\text{No. of symptomatic plants analyzed}}$ ) were determined. In each sampled plot, transects consisting of 100 plants (300 x ha) were randomly laid. Each symptomatic plant was analyzed by PCR. Our results showed that the strawberry lethal redness is present with a 62% prevalence and low incidence (0.45%) in strawberry production areas. We also demonstrate that the described symptoms are not always associated with phytoplasma infection, since only 7.96% were positive by PCR, suggesting the presence of other pathogens or abiotic stresses involved. It is necessary to continue the surveys in production areas and it would be important to study these symptoms in nurseries where strawberry seedlings are produced.

## **A VIRUS COMPLEX RESPONSIBLE FOR INCREASED EXPRESSION OF CRUMBLY FRUIT SYMPTOMS IN RED RASPBERRY ‘MEEKER’**

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Raspberry crumbly fruit is a widespread disease most commonly caused by a virus infection. In the last decade, the severity of symptoms caused by the disorder has increased considerably in commercial areas of the Pacific Northwest (PNW) of the United States and British Columbia (BC), Canada. *Raspberry bushy dwarf virus* (RBDV), a pollen-borne virus, has long been attributed as the causal agent of crumbly fruit. However, the identification of two new viruses, *Raspberry leaf mottle virus* (RLMV) and *Raspberry latent virus* (RpLV), present with a high incidence in the PNW and BC, suggested the existence of a new virus complex responsible for the increased severity of the disease. A field experiment consisting of ‘Meeker’ plants infected by single or mixed infections of RBDV, RLMV, or RpLV revealed that treatments co-infected with RBDV-RpLV-RLMV and RBDV-RLMV had a 71% and 76% reduction, respectively, in primocane growth during the establishment year. Also in the first fruiting year, plants co-infected with RBDV-RpLV-RLMV and RBDV-RpLV had the lowest berry weights, firmness and number of drupelets. Comparisons of virus titers across treatments revealed that the titer of RBDV was approximately 400-fold higher in plants co-infected with RLMV or RLMV-RpLV relative to the titer in plants infected with RBDV alone. The significant increase in the titer of RBDV in the presence of RLMV in the field was similarly found in greenhouse experiments, suggesting that environmental conditions do not have an impact on the virus interaction. Neither RBDV nor RpLV had an impact on titers of co-infecting viruses. Taken together, these findings suggest that growth reduction and severe crumbly fruit disease in ‘Meeker’ are caused by a co-infection of RBDV and RLMV or RpLV. The implications of these findings, at the applied level, are discussed in this report.

## PLANT RNA ISOLATION AID™ HELPS TO EXTRACT BETTER TOTAL RNA FROM RASPBERRIES

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### Abstract

Many plants are a big challenge for nucleic acid extraction. The most difficult ones are the woody plants which contain high concentrations of secondary metabolites, which can purify together with nucleic acids and are known inhibitors of PCR. Raspberries and blackberries are known to be very difficult plants for good quality RNA extraction. We investigated the influence of Plant RNA Isolation Aid™ (Life Technologies) on quantity of extracted total RNA (totRNA) from red raspberries. TotRNA was extracted from red raspberry leaves on MagMAX™ Express Magnetic Particle Processor using MagMAX™-96 Total RNA Isolation Kit (Life Technologies) with and without addition of Plant RNA Isolation Aid. Extracted totRNA was reverse transcribed and its quantity was checked using 18S rRNA qPCR assay. The Ct values of totRNA extraction without Plant RNA Isolation Aid™ ranged approximately between 11 and 33. The Ct values of totRNA extraction with Plant RNA Isolation Aid™ in the lysis buffer ranged approximately between 8 and 16. The results of our study show that Plant RNA isolation Aid greatly improves RNA extraction from red raspberry leaves.

### Introduction

A prerequisite for successful detection of plant RNA viruses by PCR or real-time PCR (qPCR) is a good quality RNA. Many plants are a big challenge for nucleic acid extraction due to the presence of potential PCR inhibitors. They are known to contain a lot of possible PCR inhibitors such as polysaccharides, polyphenols, proteins and other plant secondary metabolites (Osman and Rowhani, 2006; Gambino *et al.*, 2008) which can purify together with nucleic acids. Their content differs between plants, plant organs and changes during the growing period. The most difficult ones are the woody plants which contain high concentrations of secondary metabolites (Rowhani *et al.*, 1995). Raspberries and blackberries are known to be very difficult plants for good quality RNA extraction. They are known to contain large amounts of tannins in leaf tissue, which can interfere with downstream applications (Halgren, 2006).

Numerous RNA extraction methods have been used in the preparation of total RNA (totRNA) from woody plants with a more or less sufficient quantity of isolated totRNA and reduction of inhibitors, but most of them are time consuming and technically demanding (Maliyakal, 1992; Singh *et al.*, 2002; Gambino *et al.*, 2008). Recent reports have documented the benefit of RNA extraction methods from grapevines based on magnetic beads (Osman *et al.*, 2012; Rwahnih *et al.*, 2012).

Plant RNA Isolation Aid™ (Life Technologies) (PIA) is a reagent which contains among others also polyvinylpyrrolidone (PVP). The reagent is designed for the removal of common contaminants of plant RNA preparations, such as polysaccharides and polyphenolics (Applied Biosystems, 2008).

In the present work we tested an automated extraction method based on magnetic beads and investigated the influence of PIA on the quantity of extracted totRNA from red raspberries.

### **Materials and Methods**

Eighteen samples of red raspberry leaves were included in the study.

Tissue extracts were prepared by homogenizing 10-15 mg of fresh leaf tissue in 100 µL of Lysis binding solution concentrate from MagMAX™-96 Total RNA Isolation Kit (Life Technologies, Foster City, USA) or in the mixture of 90 µL of Lysis binding solution concentrate and 10 µL of PIA. The homogenization was done using TissueLyser (Retsch, Haan, Germany) shaker for 3 min at 30 Hz with two stainless steel beads of 5 mm in diameter. Extracts were centrifuged and a supernatant was used for further totRNA extraction.

50 µL of each extract were used for totRNA extraction on MagMAX™ Express Magnetic Particle Processor was used with a MagMAX™-96 Total RNA Isolation Kit (Life Technologies, Foster City, USA) according to the manufacturer's instructions. The final totRNA elution volume was 50 µL.

Extracted totRNA was reverse transcribed using a High Capacity cDNA Reverse Transcription Kit (Life Technologies) according to the manufacturer's instructions. 25 U of RNase inhibitor (Life Technologies) were added to the final volume of 25 µL. Reactions were incubated at 25 °C for 10 min, 37 °C for two hours and 85 °C for 5 min.

2 µL of cDNA of each sample were used in qPCR using assay specific for eukaryotic 18S rRNA (18S rRNA qPCR an assay, PDARS, Life Technologies). The 20 µL reactions were performed in 1X TaqMan Universal Master Mix (Life Technologies) according to the manufacturer's instructions.

10-fold serial dilutions of cDNA were prepared for detection of possible PCR inhibition in samples extracted without the addition of PIA. A PCR non-amplification control (NTC) was set up with molecular grade water instead of template.

qPCR reactions were run in duplicate for each sample on ABI PRISM 7500 (Life Technologies) using universal cycling conditions (50 °C for 2 min, 95 °C for 10 min, 45 cycles of 95 °C for 15 s and 60 °C for 1 min). Data were acquired and analyzed using the ABI PRISM 7500 Real-Time PCR System Sequence Detection System Software v1.3.

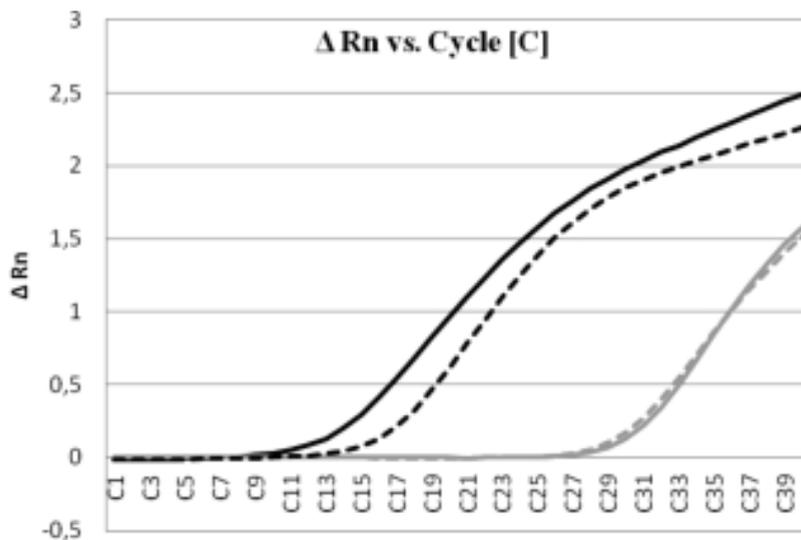


Fig. 1 - Comparison of 18S rRNA linear amplification plot of qPCR for one extracted totRNA red raspberry leaves extracted with PIA or without PIA. (— totRNA isolated with PIA; - - - 10x diluted totRNA with PIA; — totRNA isolated without PIA; - - - 10x diluted totRNA without PIA)

## Results and Discussion

In the present study we investigated the influence of PIA on the quantity of extracted totRNA.

The efficiency of extraction was tested with amplification of 18S rRNA with qPCR. The ABI Prism 7500 Real-Time PCR System Sequence Detection Software v1.3 generated fluorescence data that were used to obtain cycle threshold (Ct) values. For the final analysis the baseline was established automatically and the threshold was set at 0.1.

Big differences were observed between totRNA extractions when PIA was added to the extraction buffer. The Ct values of totRNA extraction with PIA ranged between Ct 8.33 and Ct 15.19 and for totRNA extraction without PIA between Ct 11.41 and Ct 32.46. The average Ct value of totRNA extracted with PIA was  $11.59 \pm 2.22$  and the average Ct value of totRNA extracted without PIA was  $25.50 \pm 5.09$ . The average Ct difference was  $14.77 \pm 4.93$ . The difference in Ct indicated the elimination of inhibition in samples extracted with addition of PIA. The actual differences in Ct values for the same sample ranged between 2.34 and 19.30. An example of an amplification plot for the raspberry sample is presented in Fig. 1.

The major cause of the differences is probably the elimination of different inhibitors from totRNA extracts. Woody plants are known to contain big amounts of different compounds, known to inhibit PCR. The most important ones are polyphenols and polysaccharides. Phenolic compounds are present in shoots, leaves and bud wood.

Some of them bind irreversibly to nucleic acids and co-precipitate with them (Chen *et al.*, 2000). Polysaccharides are known to be present in shoots, leaves, bulbs and tubers. They are sticky or slimy and they alter the accessibility of nucleic acids to biochemical reactants. It has been reported that even traces of polyphenolics and/or polysaccharides in RNA preparations inhibit further applications, such as RT-PCR (Dong and Dunstan, 1996; Koonjul *et al.*, 1999). It is therefore important to eliminate inhibitory compounds from RNA preparations as much as possible.

PVP is known to improve RNA extraction from woody plants when added to the extraction buffer (MacKenzie *et al.*, 1997; Dong and Dunstan, 1996). Among other components which are not known, PVP is a component of PIA, the reagent we used in the extraction buffer to improve the totRNA extraction. It has been reported that only PVP does not prevent the polysaccharide contamination of RNA extracts and better results are obtained when insoluble polyvinylpyrrolidone (PVPP) is used together with soluble PVP (Chen *et al.*, 2000). PVPP binds to polyphenolic compounds and polysaccharides and prevents their binding to nucleic acids. After binding, inhibitors are precipitated together with PVPP and RNA stays in the supernatant. Other chemicals and polymeric matrices have been reported to improve RNA extraction from plants with higher levels of inhibitors (MacKenzie *et al.*, 1997).

The results of our study show that PIA greatly improves RNA extraction from red raspberry leaves and thus potentially improves reliability of virus detection in these plants.

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## STUDIES OF *RUBUS* VIRUSES IN SLOVENIA

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In the years 2009-2011 several samples of cultivated red raspberry from different, mostly private gardens and some samples of wild blackberries were tested for the presence of viruses using ELISA and RT-PCR. ELISA was used for the detection of several nepoviruses and *Raspberry bushy dwarf virus* (RBDV), RT-PCR was used for the detection of viruses which can not be detected by ELISA. No nepovirus infections were found in analyzed samples. As expected, RBDV was the most prevalent virus, followed by *Rubus yellow net virus* (RYNV). RBDV was found only in red raspberry samples, while RYNV was found in red raspberry and blackberry samples. *Black raspberry necrosis virus* (BRNV) was found only in three samples of red raspberry from one location. These samples were also infected with RBDV, but according to the information provided by the owner, they did not show symptoms on leaves or fruits. Nine out of ten red raspberry samples from another location were infected with RBDV and RYNV. The leaves of doubly infected plants showed chlorosis and yellowing and the plants had crumbly fruit. The studies of incidence and distribution of *Rubus* viruses are still ongoing under the national research programme P2-0133.

## SURVEY OF SLRSV, RPRSV, SMYEV AND SCrV IN BELGIUM

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### Abstract

In order to assess the prevalence of strawberry viruses in Belgium, a survey was carried out since January 2011. Two aphid-transmitted viruses (SMYEV, *Strawberry mild yellow edge virus* and SCrV, *Strawberry crinkle virus*) and 2 nematode-transmitted viruses (RpRSV, *Raspberry ringspot virus* and SLRSV, *Strawberry latent ringspot virus*) were targeted through simplex and duplex PCR, derived from the protocols of Wei *et al.* (2008) and Thompson *et al.* (2003). Samples were collected mainly in strawberry fields (279 samples) but also in *Rubus* sp. (59 samples), *Ribes* sp. (32 samples) and *Prunus* sp. (95 cherry samples). SCrV analyses were limited to the genus *Fragaria*. Until now, no virus transmitted by *Xiphinema* sp. and/or *Longidorus* sp. was detected. Additionally, 5 strawberry leaf samples were infected by SMYEV two of which were also positive for SCrV. A monitoring of the infected fields has been set up.

### Introduction

*Raspberry ringspot virus* (RpRSV), *Strawberry crinkle virus* (SCrV), *Strawberry latent ringspot virus* (SLRSV) and *Strawberry mild yellow edge virus* (SMYEV) are regulated quarantine viruses that should be avoided on strawberry (and on *Rubus* sp. for RpRSV and SLRSV) plants for planting in the European Union. RpRSV and SLRSV are both nepoviruses infecting raspberries, but also strawberries and other fruit crops (Scott *et al.*, 2000; Faggioli *et al.*, 2002). These viruses are transmitted by nematodes of the genus *Longidorus* and *Xiphinema*, respectively. Dispersal of the viruses can also occur via infected seeds or mechanically (EPPO/CABI, 1997). SCrV and SMYEV are only infecting strawberry plants (*Fragaria* sp.) and are both vectored

by *Chaetosiphon fragaefolii* aphids (Posthuma *et al.*, 2000; Thompson and Jelkmann, 2004). Dispersal of both viruses can also occur mechanically, but not via seeds (EPPO/CABI, 1997). In order to assess the prevalence of these viruses in Belgium, we carried out a survey which was started in January 2011, involving sampling of plants across the Belgian territory and detection of the viruses using simplex and duplex PCR.

### Materials and Methods

Leaf samples were taken in fruit production fields across the Belgian territory. Samples were collected in strawberry fields (279 samples) but also on *Rubus* sp. (59 samples), *Ribes* sp. (32 samples) and *Prunus* sp. (95 cherry samples). Leaf samples consisted each of at minimum 10 leaves collected from different plants in the field/orchard. SCrV and SMYEV analyses were limited to the genus *Fragaria*. The two aphid-transmitted viruses (SMYEV and SCrV) and the 2 nematode-transmitted viruses (RpRSV and SLRSV) were targeted through uniplex and duplex PCR derived from the protocols of Wei *et al.* (2008) and Thompson *et al.* (2003). Extraction of viral RNA was performed using the RNeasy® Plant Mini Kit (Qiagen) and PCRs were carried out using the primer pairs RpRSV-F1/RpRSV-R1 (Ochoa-Corona *et al.*, 2006), SLRSV-F/SLRSV-R (Postman *et al.*, 2004), MKC-F/MKC-R (Klerkset *et al.*, 2004), SMYEVdeta/SMYEVdetb (Thompson *et al.*, 2003). Validation of uniplex and duplex RT-PCR for the detection of SCrV, SMYEV but also for *Strawberry mottle virus* (SMoV) was performed using strawberry positive controls used in indexing tests. Validation of RpRSV and SLRSV RT-PCR detection tests was carried out using DSMZ corresponding positive controls. The amplified DNA fragments were submitted to sequencing in order to ascertain their identity. In total, 465 samples leaf samples were tested consisting of 59 *Rubus*, 32 *Ribes*, 95 cherry and 279 strawberry samples.

### Results

Validation through sequencing of RT-PCR amplicons confirmed the infection of positive controls used in indexing tests at CRA-W since 1970s as well as the efficiency of the simplex and duplex tests set up for this survey. The status of the strawberry positive controls are shown in Tab. 1. Among the 465 tested samples, 5 strawberry samples were found positive for SMYEV two of which were also positive for SCrV.

Tab. 1 - Virus infection of strawberry positive controls used for RT-PCR validation

	SMYEV	SCrV	SMoV	RpRSV	SLRSV
V7A	-	+	-	-	-
VE7A	-	-	-	-	-
VEA	+	-	-	-	-
Kouril 172A	+	+	+	-	-
Kouril 170B	-	+	+	-	-
Kouril 171	-	+	+	-	-

### Conclusion

Uniplex and duplex RT-PCR identification tests were set up and validated for 5 strawberry viruses (SCrV, SMEYV, SMoV, RpRSV and SLRSV). The viral status of positive controls used in strawberry indexing tests at CRA-W since the 1970s was confirmed by PCR and sequencing. The survey conducted across the Belgian territory on cherry, red currant, raspberry and strawberry fields allowed to find out, among the 465 samples tested, 3 SMYEV and 2 SMYEV-SCrV positive samples on strawberry. In order to follow the infections, a monitoring consisting of insect trapping, weeds testing and random sampling was set up.

### Acknowledgment

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## SPREAD OF *HOP LATENT VIROID* (HLVd) IN HOP GARDEN

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Hop (*Humulus lupulus L.*), is a vegetative propagated monoculture grown for many years at the same location, and is severely endangered with virus and viroid infections. Productive potential is decreased by these pathogens which is observable not only on the yield but on the quality of hop cones as well. Cones are used mainly in beer production. Very important is content of hop resins and essential oil for the aroma and taste of beer. It is the reason why many countries have begun a recovery process. Viroids are the smallest autonomously replicating pathogenic agent described to date. Two viroids are present in hop plants: *Hop latent viroid* (HLVd) and *Hop stunt viroid* (HSVd). Analyses of commercial hop gardens in Czech Republic shown very high infection of HLVd, and viroid infection decreased content of alpha bitter acids in cones on average by 40%. In Czech Republic the main variety is Saaz semi-early red-bine hop, which is the typical fine aroma hops, grown in the regions of Žatec (Saaz), Ústěk (Auscha) and Tršice (Trsitz) as Oswald's clone 31, 72 and 114. In period 2004-2010 we collected samples from experimental hop garden planting from virus-free and viroid-free rootstocks and analyzed spreading of reinfection by HLVd in cones of Oswald's clone 31, 72, and 114. No morphological symptoms were observed on infected plants. These results are important for elimination of HLVd and production virus and viroid free planting material of hop in Czech Republic.

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## A NEW *FLEXIVIRUS* IDENTIFIED IN BLACKBERRY

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Two symptomatic smooth blackberry (*Rubus canadensis*) plants, collected during the survey on plant viruses in the Great Smoky Mountains, contained high-molecular-weight dsRNAs indicative of virus infections. Purified dsRNAs were used as a template for shotgun cloning and sequencing. Analysis of clones revealed the presence of *Blackberry yellow vein-associated virus* (BYVaV) and *Blackberry virus S* (BIVS), but also a putative new flexivirus. This prompted further molecular characterization of this virus, tentatively named *Rubus canadensis virus-1* (RuCV-1). The RuCV-1 genome is 8.3 kb, polyadenylated and codes for five ORFs: replication-associated polyprotein (RAP), triple gene block (TGB) and viral coat protein (CP). Phylogenetic analyses of the entire RAP indicated that RuCV-1 is closely related to members of the genus *Foveavirus* (fam. *Betaflexiviridae*). However, identities with extant foveaviruses are below the current ICTV species demarcation thresholds, suggesting that RuCV-1 is a novel member of the taxon. A virus-specific RT-PCR based method has been developed and study on the incidence/importance of this virus in cultivated blackberries in south-eastern United States is ongoing.

## A PUTATIVE NEW *EMARAVIRUS* ASSOCIATED WITH BLACKBERRY YELLOW VEIN DISEASE

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### Abstract

Blackberry yellow vein disease complex (BYVD) is a major concern to fresh market blackberry production in the United States. The disease is caused by the synergistic effects of multiple virus infections. The identity of the viruses infecting plants is usually insignificant and symptom severity is closely linked to the number of viruses infecting plants. A new multipartite negative sense RNA virus, a member of the genus *Emaravirus*, was identified in BYVD plants showing leaf mottling, chlorotic ringspots and curved midribs. RT-PCR detection protocols have been developed and used to evaluate virus distribution in the Southeastern United States in both wild and cultivated blackberries. The infection rate was higher in the former, indicating that the virus is well established in the area. Studies on the population structure of isolates collected from five US states showed geographically-related clustering, suggesting that virus movement because of infected propagation material is rather minimal.

### Introduction

Blackberry yellow vein disease (BYVD), a disorder caused by virus complexes, is the most important virus disease affecting blackberry production in south-eastern United States. The disease is characterized by yellowing, leaf distortion, and sometimes line patterns on the leaves (Susaimuthu *et al.*, 2008). Affected areas may turn necrotic and, in severe cases, BYVD may lead to plant death. The most significant impact of BYVD on blackberry is a decline in productivity that can lead to a 5-7 year rotation. To date at least fifteen viruses have been found associated with blackberry yellow vein disease, majority of which were previously unknown (Martin *et al.*, 2012). Single infections are asymptomatic but several BYVD plants were infected with only one of the viruses previously identified, suggesting the presence of other, unknown, viruses that act synergistically to cause disease. The objective of this work is to identify and characterize unknown virus(es) and possible vector may be involved in the etiology of BYVD. In this study, we report work on the characterization of a new emaravirus, provisionally named *Blackberry leaf mottle associated virus* (BLMaV), identified in blackberries exhibiting BYVD symptoms.

## Materials and Methods

Potted blackberries of cv. Natchez, used as sentinel plants to monitor vectors and virus movement in a blackberry field heavily infested with BYVD, were replaced in monthly intervals through the period spanning the blackberry-growing season (April-October) and then tested in the lab for virus infections. Double-stranded RNA (dsRNA) was extracted from 20g of blackberry leaf tissue with virus-like symptoms as previously described (Tzanetakis and Martin, 2008). Degenerate oligonucleotide-primed reverse transcriptase-PCR (DOP RT-PCR) was used for amplification of the purified dsRNA template. Material was subjected to high throughput sequencing using the Illumina platform as previously described (Laney *et al.*, 2011). RNA1 sequence was obtained by combining degenerate primers and emaravirus terminal sequences. The complete viral RNA2, 3, and 4 sequences were obtained as previously described (Laney *et al.*, 2011). The short reading sequences were assembled using Velvet (Zerbino and Birney, 2008) and CAP3 (Huang and Madan, 1999). Contigs were then searched against NCBI database using BlastX. For BLMaV detection, RT-PCR test was developed using virus-specific set of primers designed to amplify a 600 bp portion of genomic RNA2.

Phylogenetic analysis was done using the Neighbor-Joining method using MEGA 5.0 (Tamura *et al.*, 2011). The occurrence of BLMaV was investigated using RT-PCR in blackberry samples showing BYVD symptoms collected from south-east United State (AR, FL, GA, MS, NC, and SC).

## Results

Several sentinel virus-tested blackberries exhibited virus-like symptoms after only one month of exposure in the open field. Symptoms included leaf mottling, ring spots, oak line pattern, and yellow veins (Fig.1). Sequencing of randomly amplified cDNAs derived from dsRNA templates yielded a clone with homology to the glycoprotein precursor of bunyaviruses. RT-PCR was developed and optimized for detection the virus in infected blackberries. Amplicons of expected size were obtained and specificity of RT-PCR was confirmed by sequencing. The virus was detected in several sentinel blackberries, suggesting that there is an active vector transmitting the virus to healthy blackberries.

Occurrence of the BLMaV was investigated in samples collected from south-eastern United State. The virus found in samples collected from all states (AR, FL, GA, MS, NC, and SC) and was present in over 30% of the field grown blackberry samples tested. The infection rate was even higher in tested wild blackberry samples, exceeding 50%.

Four RNAs have been identified to date and sequence completed. Predicted translation products of these RNAs share similarities with *Fig mosaic virus* (FMV) and *Rose rosette virus* (RRV), tentative members in the genus *Emaravirus*. Phylogenetic analysis of the nucleocapsid protein of BLMaV and orthologs of selected species in the *Bunyaviridae* revealed consistent clustering of the virus within the *Emaravirus*

genus clade with high bootstrap values (Fig.2), depicting the genetic relatedness between BLMaV and members of the genus *Emaravirus*.

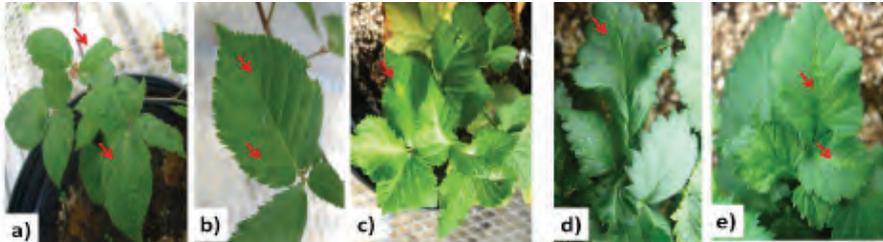


Fig. 1 - Symptoms associated with BLMaV infections: a) Leaf mottling and curved midribs; b) Chlorotic spots; c) Vein yellowing; d) Oak line pattern; e) Yellow feathering.

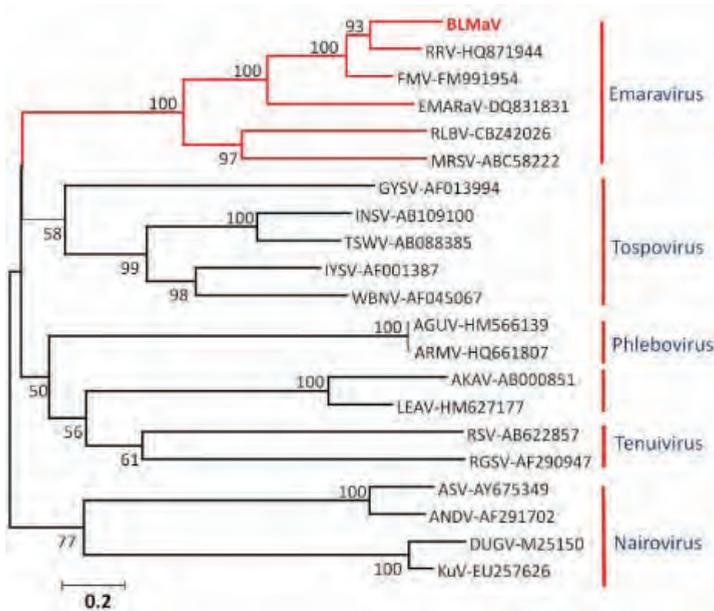


Fig. 2 - Unrooted phylogenetic tree constructed with the complete amino acid sequences of BLMaV p3 and orthologs of representative members of the family *Bunyaviridae* and genus *Tenuivirus*.

## Discussion

In this study, we have identified a new emaravirus associated with blackberry yellow vein disease, a disorder caused by the interaction of several viruses infecting the crop (Martin *et al.*, 2012). Emergence of new emaraviruses may be the result of increased trade and/or movement of infected plants into new geographical areas.

The BLMaV sequence data suggest that the virus should be considered a new member of the genus *Emaravirus* as it shares limited levels of identity with members of this taxon. Eriophyid mites transmit all the other members of the genus, e.g. *European mountain ash ringspot associated virus* (EMARaV), FMV, RRV, and *Raspberry leaf blotch virus* (RLBV) (Amrine *et al.*, 1988; Mielke *et al.*, 2007; Elbeaino *et al.*, 2009; and McGavin *et al.*, 2012). Accordingly, we suspect eriophyid mites as possible vectors of BLMaV. Transmission experiments are currently underway in order to identify vector species involved in the epidemiology of the virus. In addition, and likewise plant viruses, BLMaV will be present in all blackberry progeny derived clonally from infected mother plants, indicating the importance of virus-tested plant material. Finally, the virus was found in over 50% of tested wild blackberry samples underlying the importance of wild blackberries as inoculum sources.

The most important measure for containment blackberry viruses including BLMaV is the development of reliable detection methods, enabling for early detection and virus elimination. We aim to obtain sequence information from several isolates so as to develop primers for the most conserved regions of the virus assuring detection of all virus variants.

## Acknowledgements

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## **DETECTION OF *BLACKBERRY CHLOROTIC RINGSPOT VIRUS*, *STRAWBERRY NECROTIC SHOCK VIRUS* AND *BLACKBERRY YELLOW VEIN-ASSOCIATED VIRUS* BY TAQMAN qPCR**

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### **Abstract**

Three TaqMan quantitative real-time PCR assays with absolute quantification for the detection of *Blackberry chlorotic ringspot virus* (BCRV), *Strawberry necrotic shock virus* (SNSV), and *Blackberry yellow vein-associated virus* (BYVaV) have been developed. The assays were applied successfully on plants tested positive with BCRV, SNSV or BYVaV by conventional PCR. Sanger sequencing confirmed that the qPCR DNA amplicons were of viral origin. Standard curves were constructed for the three assays and the detection limit was determined. Quantification data showed reproducible results for both Cq values and calculated copy numbers. Serial dilution tests indicated that the qPCR assays were more sensitive compared to conventional PCR.

### **Introduction**

*Rubus* and *Fragaria* species are affected by more than 30 virus and virus-like diseases. In this pool of pathogens, *Blackberry chlorotic ringspot virus* (BCRV) (Tzanetakis, Martin, and Scott, 2010), *Strawberry necrotic shock virus* (SNSV) (Tzanetakis, Mackey, and Martin, 2004a) and *Blackberry yellow vein associated virus* (BYVaV) (Tzanetakis *et al.*, 2006b) are some of the most widespread viruses. BCRV and BYVaV are associated with blackberry yellow vein disease, an emerging problem in the US initially suspected to be caused by *Tobacco ringspot virus* but later confirmed as a complex of several viruses (Tzanetakis *et al.*, 2006b). Similarly, SNSV until few years ago was grouped as a strain of *Tobacco streak virus* (TSV) (Fulton, 1970) and only recent data have established its status as a separate species.

BCRV and SNSV are members of the tripartite genus *Ilarvirus*, family *Bromoviridae*. There are 19 recognized *Ilarvirus* species to date, and the genus is classified into 6 subgroups based on serological relationships (King *et al.*, 2011). Subgroup 1 contains four members (Tzanetakis *et al.*, 2010) including BCRV and

SNSV. Their genomic RNA 1 is translated to a polyprotein with methyltransferase and helicase domains, whereas RNA 2 encodes an RNA dependent RNA polymerase and a protein that is a putative suppressor of RNA silencing based on homology with 2b protein of *Cucumovirus* (Lucy *et al.*, 2000). RNA 3 encodes the movement and coat proteins (MP and CP respectively), the latter being expressed via a subgenomic RNA 4 (King *et al.*, 2011).

The genome sequences of BYVaV have been published (Tzanetakis *et al.*, 2006b) and the virus has been classified as a member of the genus *Crinivirus*, family *Closteroviridae*. RNA 1 is 7,800 nucleotides long and has motifs of papain-like protease, methyltransferase, RNA helicase and RNA-dependent RNA polymerase. RNA 2 is 7,916 nucleotides long and encodes nine open reading frames involved in virion assembly, vector transmission, movement, protection and other functions yet to be determined (Poudel *et al.*, 2012).

As knowledge about the population structure of BCRV, SNSV and BYVaV has increased substantially, there has been a need for more accurate and sensitive methods to diagnose these important berry pathogens. For that reason, this study presents the development of three TaqMan® quantitative PCR (qPCR) assays to detect BCRV, SNSV and BYVaV. The assays displayed better sensitivity than any other detection methods available and were able to calculate exact virus copy number in infected plant materials.

## Materials and Methods

**Total RNA extraction and cDNA synthesis:** Infected leaf tissue was used for RNA extraction as described previously (Tzanetakis *et al.*, 2007). Briefly, 50 mg leaf tissue from virus-infected plant was ground in 1 ml RNA extraction buffer (200 mM Tris-HCl pH 8.5, 300 mM lithium chloride, 1.5% lithium dodecylsulphate, 10 mM EDTA, 1% sodium deoxycholate, 1% NP-40 and 1% 14 M  $\beta$ -mercaptoethanol solution (vol/vol) added just before use). An equal amount of 5.8 M potassium acetate (3.8 M potassium, 5.8 M acetate) was added to the mix (600  $\mu$ l) and centrifuged at 16,000 x g for 10 min. Seven hundred fifty  $\mu$ l of the supernatant were mixed with an equal volume of 100% isopropanol and chilled at -20°C for at least 30 min. The mix was then centrifuged for 20 min and the resulting pellet was re-suspended in 500  $\mu$ l wash buffer (10 mM Tris-HCl pH 7.5, 0.5 mM EDTA, 50 mM NaCl, 50% ethanol) and 20  $\mu$ l of silica/glass milk were added. The mix was pulse centrifuged for 10 sec at 9,400 x g and washed twice to eliminate inhibitors. After the final wash, the mixture was centrifuged for 2 min at 16,000 x g and pellet was dried in a speedvac. The pellet was re-suspended in 150  $\mu$ l TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA) and centrifuged for 2 min at 13,500 x g. 100  $\mu$ l of the supernatant containing the RNA was collected and stored at -80°C for subsequent experiments. RNA concentration was measured using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific) and normalized to 100 ng/ $\mu$ l. Reverse transcription (RT) was conducted with necessary positive, negative and water controls also as described (Tzanetakis *et al.*, 2007). Briefly, 250 ng of purified nucleic acid, 1.5  $\mu$ g random hexameric primer and 0.4 mM virus genus-specific primers (Tab. 1)

were used. 50 U Maxima<sup>TM</sup> Reverse Transcriptase (Fermentas) were added with 6 U RiboLock RNase Inhibitor (Fermentas), 0.4 mM dNTPs, 5 µl 5x reverse transcriptase buffer (250 mM Tris-HCl pH 8.3, 375 mM KCl, 15 mM MgCl<sub>2</sub>, 50 mM DTT) and water to 25 µl. The RT mix was incubated first at room temperature for 2 min and then at 50°C for 75 min followed by 5 min inactivation at 85°C. The cDNA product was stored at -20°C until use.

**TaqMan probe and primer design:** Nucleotide sequences from RNA 2 of BYVaV and RNA 3 of BCRV and SNSV (Poudel *et al.*, 2012; Tzanetakis *et al.*, 2010) were introduced to the web-based RealTimePCR tool (Integrated DNA Technologies) to generate possible qPCR probe-primer sets. These provisional nucleotide sets were manually aligned against local viral sequences using the built-in ClustalW Multiple alignment option (Thompson, Higgins, and Gibson, 1994) of BioEdit (Hall, 1999) to confirm sequence homology and position along the virus genomes. Physical properties of probes and primers were manually checked using OligoAnalyzer tool (IDT, version 3.1). Acceptable probe-primer sets were then synthesized by IDT as PrimeTime qPCR Mini Assays with a fluorophore group (FAM®) at the 5' end, a quencher group (3' Iowa Black® FQ) at the 3' end, and an additional quencher (ZEN®) at the 10<sup>th</sup> nucleotide from the 5' end to reduce background emission noise of the assays. Working concentrations of probes and primers were set at 250 and 500 nM, respectively.

**TaqMan real-time PCR and construction of standard curves:** Quantitative PCR assays were performed with 5% cDNA in 20 µl reactions using the 2x Universal Master Mix (ABI) and 10x PrimeTime qPCR Assays probe-primer mix (IDT). A CFX96 qPCR thermocycler (BioRad) was used to run the reactions comprising of 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 60 sec. After positive qPCR results were achieved, conventional PCRs were conducted using primers identical to qPCR forward and reverse primers (Tab. 1) to amplify the qPCR products for the development of standard curves. Briefly, the qPCR products were diluted 1,000 times in water and 2.5 µl of the diluted DNA were used in 50 µl PCR reactions using 10x Taq Buffer (500 mM KCl, 100 mM Tris HCl pH 9.0, 15 mM MgCl<sub>2</sub>, and 1% Triton X-100, GenScript), 0.4 mM of 5' and 3' primers, 0.2 mM dNTPs and 1.25 U Taq DNA polymerase (GenScript). The initial denaturation was set at 94°C for 2 min. Cyclic conditions consisted of 40 cycles at 94°C for 15 sec, 55°C for 15 sec, and 72°C for 30 sec, followed by final extension at 72°C for 10 min. Negative and water controls were included in each run accordingly. The PCR products were electrophoresed on a 3.5% agarose gel, stained in GelRed<sup>TM</sup> solution (Biotium) and visualized in a UV transilluminator. DNA bands of expected amplicon sizes were cloned into TOPO-TA pCR 2.1 vector (Invitrogen) according to manufacturer's protocol. The vector was transformed into  $\alpha$ -Select Chemically Competent Cells (Bioline), and individual white colonies were grown in 5 ml LB broth Ampicillin overnight at 37°C in shaking condition. Plasmid DNA was extracted using GeneJET<sup>TM</sup> Plasmid Miniprep Kit (Fermentas) according to the manufacturer's protocol, and Sanger sequencing was performed to confirm the identity of the plasmid inserts. The plasmid DNA concentrations were measured in a NanoDrop 1000 and 10-fold serial dilutions from 3x10<sup>7</sup> to 3x10<sup>0</sup> plasmid copies/µl were prepared. Standard curves were

constructed from the plasmid dilution and used for calculating copy numbers.

**Comparison with conventional PCR detection primers:** Detection primers for BCRV, SNSV and BYVaV (Tab. 1) were employed to amplify virus DNA from the same cDNA samples used for developing the qPCR assays to compare the sensitivity between the two tests. For this purpose, cDNA samples were diluted by 8-fold corresponding to 3 PCR cycles. PCR conditions were as published for each primer set (Susaimuthu *et al.*, 2008; Tzanetakis, Gergerich, and Martin, 2006a; Tzanetakis, *et al.*, Mackey, and Martin, 2004b) and qPCR conditions as described earlier.

Tab. 1. List of oligos used in this study

Primer name	Nucleotide sequence 5' → 3'	Cq value in detection	Standard curve's limit of detection (plasmid DNA copies)
Random hexamer	NNN NNN		
Ilarsub1	GCA TCT C		
CrimIR1	TTT TYGAGW TTT TAW AAT ACA		
BCRV PCR detection (Tzanetakakis <i>et al.</i> , 2006a)	Forward primer: GTT TCC TGT GCT CCT CA Reverse primer: GTC ACA CCG AGG TAC T		
SNSV PCR detection (Tzanetakakis <i>et al.</i> , 2004b)	Forward primer: AGT ATT TCT GTA GTG AAT TCT TGG A Reverse primer: ATT ATT CTT AAT GTG AGG CAA CTC G		
BYVaV PCR detection (Susaimuthu <i>et al.</i> , 2008)	Forward primer: CGG TGT CCT CAA GAT GTT TGT Reverse primer: TGG TTG TGT GAA GGC AAT GTA Forward primer: GCT GCT TCT TGA GCT TAC CTG Reverse primer: CCG TTG GAG TTA CCA TTT CTT Probe: <sup>56</sup> -FAM\CC AGA CCA A\ZEN\C CAT CCAACG CC\3IABkFQ\ Forward primer: CGA TGC TAT CAC GTT GAA GAC	21.15	30
BCRV assay	Reverse primer: ACC AAA CCA AAT GTC CCA TC Probe: <sup>56</sup> -FAM\TT CCC GAA A\Zen\T CAA TGA TGA CAC GAA \3IABkFQ\ Forward primer: TTG ATA GAA GCG AGG TTAARA CC	20.93	30
SNSV assay	Reverse primer: GGT AAC ATA CTG AGG TCT AAT CTT CC Probe: <sup>56</sup> -FAM\AA AAG ATG G\ZEN\G TTG GAG TGG ACA GGA C\3IABkFQ\ Forward primer: TTG ATA GAA GCG AGG TTAARA CC	19.46	30

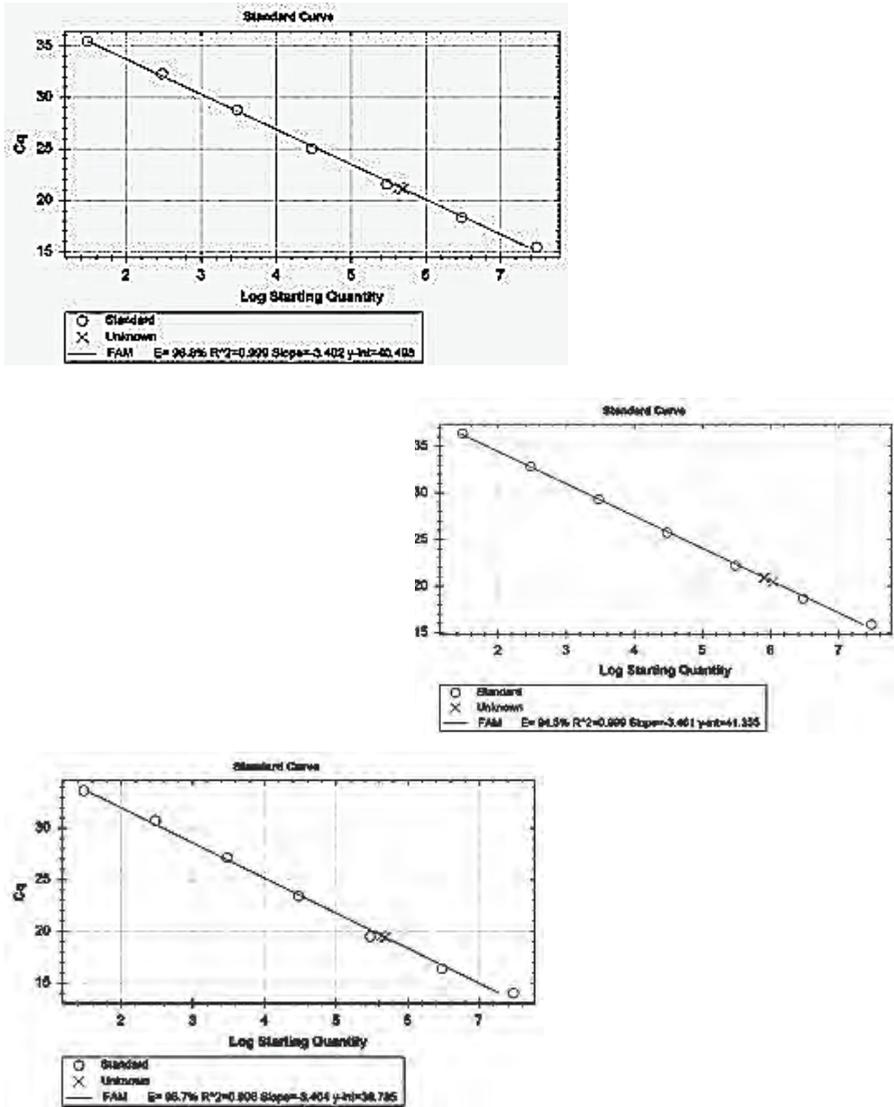


Fig. 1 - Standard curves of the developed TaqMan qPCR assays. (A) BCRV; (B) SNSV; (C) BYVaV. Circles and cross marks represent plasmid DNA dilutions and virus copy number in plant samples, respectively. E = efficiency; R<sup>2</sup> = correlation coefficient; y-int = y-intercepts.

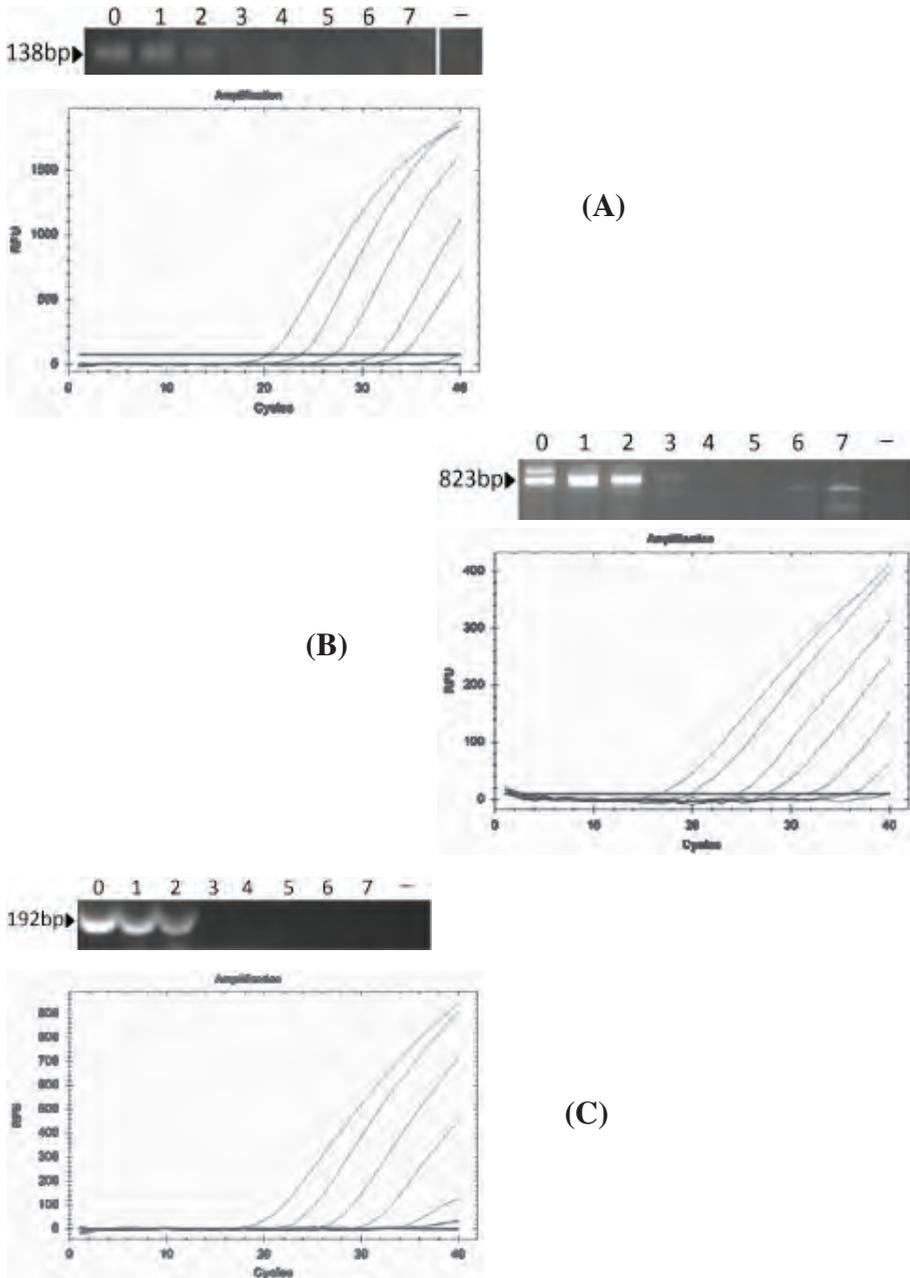


Figure 2. PCR and qPCR detections of (A) BCRV, (B) SNSV and (C) BYVaV. The cDNA samples were serially diluted in 8-fold, corresponding to 3 PCR cycles. Nucleotide sequences are shown in Tab. 1.

## Results and Discussion

**Properties of the qPCR assays:** To develop qPCR assays to detect BCRV, SNSV and BYVaV isolates, data compiled in previous studies were used (Tzanetakis et al., 2010; Tzanetakis et al., 2006b). Based on these sequence libraries, four qPCR probe-primer sets were designed and synthesized for each virus. Depending on the consistency of their  $C_q$  values, intensity and noise of fluorescence signals in detecting virus from plant samples, two probe-primer sets for each virus species were selected and their qPCR products were re-amplified with a conventional PCR. DNA bands of correct sizes were cloned to TOPO TA 2.1 plasmid vector for sequencing which confirmed that the bands were of viral origin., and Standard curves were constructed from purified plasmid DNA serially diluted from  $3 \times 10^7$  to  $3 \times 10^0$  copies/ $\mu$ l. Three probe-primer sets (Table 1) with qPCR properties within recommended qualities for TaqMan assays showed efficiencies (E) between 90%-110%, correlation coefficients ( $R^2$ ) more than 0.990, slopes from -3.10 to -3.60, and y-intercepts close to 40 (Figure 1). Exact viral copy numbers in corresponding plant samples were also calculated by plotting against the standard curves (Table 1).

**Detection limits of qPCR assays and normal PCR detection primers:** Detection primers for BCRV, SNSV and BYVaV (Table 1) were used to amplify virus DNA from the same cDNA samples used for developing the qPCR assays. Whereas BYVaV and SNSV detection primers (Susaimuthu et al., 2008; Tzanetakis et al., 2004a) worked consistently (Figure 2), BCRV primers (Tzanetakis et al., 2006a) did not because of mismatches of local virus genome sequence to the published detection primer set (data not shown). For that reason, BCRV primers identical to those of the qPCR were used for comparison (Table 1). Using an 8-fold serial dilution (corresponding to three PCR cycles) of the infected plant cDNA, BCRV PCR primers only displayed band until the 2nd dilution, whereas BCRV qPCR assay could detect positive signal at the 4th dilution (64x more sensitive). SNSV and BYVaV detection primers showed PCR bands of correct sizes until the 2nd dilution, whereas their corresponding qPCR assays still displayed signals at the 5th dilution (512x more sensitive). BCRV, SNSV and BYVaV are major threats to berry fruit industry in the US and also around the world. Due to the addition of RNA sequencing data, it was necessary to develop new and sensitive methods to detect these viruses. Three separate TaqMan real-time RT-PCR detection assays were developed for BCRV, SNSV and BYVaV. Standard curves showed that the assays were reliable and replicable to detect virus infection in plant leaf tissues. Using serially diluted cDNA samples, the qPCR assays showed better sensitivity than conventional PCR. Better sensitivity and the ability to calculate exact virus copy numbers have made these assays attractive tools in blackberry virus diagnosis.

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## GENOME ORGANIZATION AND SEQUENCE DIVERSITY OF A NOVEL BLACKBERRY AMPELOVIRUS

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### Abstract

Blackberry yellow vein disease (BYVD) is an important viral disease in blackberry. Recently, several new viruses associated with BYVD have been identified, one of which is the subject of this communication. Experiments were conducted to characterize the virus, determine its geographic distribution and population structure in the US. Phylogenetic analysis revealed the close relationship of the virus with *Grapevine leafroll associated virus-3*, the type member of genus *Ampelovirus* in the family *Closteroviridae*. Analysis of nucleotide and amino acid diversities among the US isolates revealed considerable variations in all three regions studied.



Fig.1. Symptoms observed on ampelovirus-infected blackberry plant.

### Introduction

Blackberry yellow vein disease (BYVD) is the most important disease in fresh market blackberries in the southern United States. Symptoms include oak-leaf patterns, irregular chlorosis, line patterns, yellowing of veins, and ultimately dieback resulting in substantial yield losses (Susaimuthu *et al.*, 2006; 2007). Symptom onset is associated with the presence of multiple viruses and often single infections are symptomless. *Blackberry yellow vein-associated virus*, *Blackberry virus*

*Y, Beet pseudo-yellows virus, Blackberry chlorotic ringspot virus, Blackberry virus E, Blackberry virus S, Impatiens necrotic spot virus, and Tobacco ringspot virus* have often been found associated with BYVD (Martin *et al.*, 2012). A new closterovirus was identified in symptomatic plants in Mississippi (Sabanadzovic *et al.*, 2011) (Fig. 1). This paper discusses the molecular characterization and population structure of this virus.

## Materials and Methods

**Identification and characterization:** Double-stranded RNA from infected plants was extracted with protocol involving selective chromatography (Valverde, 1990), and used as a template for further molecular work. Virus sequences were obtained by random-primed cloning and next-generation sequencing using the Illumina platform as previously described (Laney *et al.*, 2011). Terminal sequences of the virus genome were determined by 5' and 3' RACE (Invitrogen). The relationship of the virus with other members of the family *Closteroviridae* was determined after phylogenetic analysis of heat shock protein 70 homolog (HSP70h) sequences using MEGA5 software (Tamura *et al.*, 2011).

**Genetic diversity:** Blackberry samples collected between 2008-2011 were screened for the presence of the virus and total of twenty five isolates obtained from Arkansas, Georgia, Mississippi, North and South Carolina were used for the diversity analysis. Total nucleic acids were extracted from 50 mg tissue (Tzanetakis *et al.*, 2007), and reverse transcribed using random primers. Primers were designed to amplify ~1,200 nucleotide (nt) region of cDNAs in polyprotein (region between methyltransferase and helicase domains), HSP70h, and minor coat protein (CPm) genes (Tab. 1) using Phire Hot Start II DNA Polymerase (New England Biolabs) by two-step PCR. The samples were subjected to an initial denaturation at 98<sup>o</sup> C for 30 s, followed by 35 cycles of denaturation at 98<sup>o</sup> C for 5 s and annealing and extension at 72<sup>o</sup> C for 30 s, and a final extension at 72<sup>o</sup> C for 1 min. The PCR products were purified using the GeneJet PCR purification kit (Fermentas Life Sciences), added 'A' overhang using *Taq* polymerase, purified, and cloned into Topo 2.1 vector according to manufacturer's protocol (TOPO-TA cloning Kit, Invitrogen). The  $\alpha$ -select chemically competent *Escherichia coli* cells (Bioline) were transformed with the cloning mixture according to the protocol and transformed colonies were grown on Luria-Bertani (LB) plate containing 50  $\mu$ g/  $\mu$ l Kanamycin and 40  $\mu$ M Bromo-chloro-indolyl-gaactopyranoside (X-gal). The white colonies were screened by colony PCRs with gene-specific primers and *Taq* DNA polymerase (GenScript). Thermal cycler program consisted of 3 min initial denaturation step at 94<sup>o</sup> C, followed by 40 cycles of 30 s denaturation at 94<sup>o</sup> C, 20 s annealing at 54<sup>o</sup> C and 1 min 30 s extension at 72<sup>o</sup> C. The recombinant colonies were sequenced at the Functional Biosciences facilities at Wisconsin with M13 forward and reverse primers. Sequences were edited using the Sequence Scanner Software v1.0 (Applied Biosystems) and assembled by CAP3 (Huang and Madan, 1999). Variation in nt and predicted amino acids (aa) sequences were determined with ClustalW.

Tab. 1 - Primer sequences used for the amplification of genes for diversity analysis.

Primer name	Primer sequences	Position
CPm-1F	5'- GACGAATTCGAGGAGAGAGAGGCTT-3'	+ strand
CPm-2F	5'- AGGGTAGACAATTTTCGCGGTTGT-3'	+ strand
CPm-1R	5'- AGGCACTGGCCAAAGCGTTAGC-3'	- strand
CPm-2R	5'- ACTGCTCCTCCCTCACCTTT-3'	- strand
CPm-3R	5'- TATACTGCTCCTCCCTCACCT-3'	- strand
HSP 70h-1F	5'- GGACGTGGGCATAGACTTCGG -3'	+ strand
HSP 70h-2F	5'- TGCTATTCGGCGTCGGGTGC-3'	+ strand
HSP 70h-3F	5'- TGCAGGAGGTTGTACCAGGG-3'	+ strand
HSP 70h-4F	5'- CAGGGTGGCAGGTAGCATCTT -3'	+ strand
HSP 70h-1R	5'- CTGAACGTTAGTTCGTTGAGGTAACACGA -3'	- strand
HSP 70h-2R	5'- CGTACTTAACCGTATCCGTTCTCTTGCC-3'	- strand
HSP 70h-3R	5'- TCAGCGTCCCATCGAGCGAT-3'	- strand
HSP 70h-4R	5'- GCCATTGATAGTGACGCTCAGCG -3'	- strand
Polyprotein-1F	5'- TGGTGTGCCCTTTCGCGGCAC-3'	+ strand
Polyprotein-2F	5'- TCGGGCTGTTGTCAGTGGAT-3'	+ strand
Polyprotein-3F	5'- GGTGCTTTTCGGCGATCTTGTGG-3'	+ strand
Polyprotein-1R	5'- CTAGCTAAGCCAGCAACACCCAC-3'	- strand
Polyprotein-2R	5'- AAGTCTGACCTCCCCACTCC-3'	- strand
Polyprotein-3R	5'- CACCGATGCAGGGAGAAGTCTG-3'	- strand
Polyprotein-4R	5'- CAACTCCCCACACCGATGCAGG-3'	- strand

HSP70h- Heat shock protein 70 homolog      CPm- minor coat protein

## Results and Discussion

Genome sequence analysis revealed that the new virus belongs to the genus *Ampelovirus*, family *Closteroviridae*. The genome is approximately 18.5 kb long and resembles *Grapevine leafroll-associated virus 3* (GLRaV-3) in organization. ORF 1a contains conserved domains of protease, methyl transferase, AlkB, and helicase, whereas ORF 1b encodes the RNA-dependent RNA polymerase (RdRp). ORFs 2-7 encode two small proteins with transmembrane motifs, HSP70h, coat protein homolog (CPh), major coat protein (CP), and CPm, respectively. A putative RNA silencing protein is encoded by ORF 8. The proteins encoded by the 3'-proximal ORFs do not show significant similarity to known viral proteins. Phylogenetic tree constructed using the sequences of HSP70h showed the close relationship of the new virus with members of the *Ampelovirus* Subgroup II, and in particular with GLRaV-3 (Fig. 2). The RdRp is 65% identical with the GLRaV-3 ortholog, whereas the CP (34 kDa) and CPm (54 kDa) are 53% and 34% identical with their counterparts in GLRaV-3 and



universal conventional and real-time PCR detection assays will be developed based on the sequence information obtained from this study to detect a wide range of isolates which would in turn minimize movement of the virus via propagative material.

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## TRANSGENIC APPROACH FOR IMPROVING RESISTANCE OF PLUM CULTIVARS FOR SHARKA DISEASE

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### Abstract

Since the first record of Sharka disease in Bulgaria, the disease has progressively spread via infected plant materials throughout Europe where it has destroyed well over 100 million stone fruit trees. The disease has serious agronomic and political consequences due to the enormous economic losses. Because only few PPV resistance genes have been found to naturally occur in *Prunus*, scientists have utilized genetic engineering techniques to develop resistant plums by inserting specific genes from the PPV genome into the DNA of *Prunus* host plants. For improving the plants resistance to *Plum pox virus* (PPV) we used two technologies based on co-suppression gene and RNA-silencing. Binary vector pCamPPVcp which contained PPV-CP gene in sense-orientation and self-complementary fragments of gene PPV-CP were used for realization post-transcriptional gene silencing. Seven independent transgenic lines with PPV-CP gene and five transgenic lines with a two inverted repeats of PPV-CP gene fragment were produced in our laboratory. Field testing of resulted transgenic plants and “Honey Sweet” confirmed efficiency of usage RNAi strategy for protection stone fruits from Sharka disease.

### Introduction

Sharka is the most devastating disease of stone fruits trees and its occurrence worldwide has serious agronomic, economic and political consequences. The reduced fruit quality and premature fruit drops caused by Sharka disease make stone fruit production in Europe problematic. Because only few PPV resistance genes have been found to naturally occur in *Prunus*, scientists have utilized genetic engineering techniques to develop resistant plums by inserting specific genes from the PPV genome into the DNA of *Prunus* host plants.

## Materials and Methods

Transgenic plants of clones PPVcp7 with PPV-CP gene in sense orientation, RNAi6 contains self-complementary fragments of PPV-CP gene, no transformed plants of cultivar Startovaya and plants of ‘Honey Sweet’ were used for testing PPV resistance. Clone PPVcp7 didn’t infect in previous attempt (Mikhailov, R.V., *et al.* 2012), RNAi6 is a new clone transformed by pCamPPVRNAi (Mikhaylov, R.V. and Dolgov, S.V. 2011). Three plants of each clone were infected by grafting of buds from bud stick with a high level of PPV infection detected by Western blotting. Amplification of HC-Pro gene and 3’-untranslated region of virus genome were used for PPV-Detection. Western-blot analysis was also performed using rabbit polyclonal antibodies to PPV coat protein (Loewe).

## Results and Discussion

Western Blot analysis of plants grafted by PPV infected buds showed the presence of lines corresponding PPV coat protein in all control and PPV-CP transformed plants of clone PPVcp7, were observed in samples from plants transformed ‘hairpin’ construct (RNAi6) and ‘Honey Sweet’. Similar results on the same plants were observed by amplification of the 412bp fragment of HC-Pro PPV gene and 245bp fragment of 3’-untranslated region of virus genome (Tab. 1). This results confirmed efficiency of RNAi strategy for protection stone fruits from PPV virus attack, and susceptibility of plants expressed PPV-CP gene in sense orientation.

Tab. 1- Analysis of transgenic plants with different expression cassettes for PPV presence after artificial inoculation by virus.

Cultivar, transgenic line	Infected plants	Plants with visual symptoms	Positive PCR assay		Western blot
			3’UTR	HC-Pro	
Startovaya control	3	3	3	3	3
Startovaya PPVcp7	3	2	3	3	3
Startovaya RNAi6	3	-	-	-	-
‘Honey Sweet’	3	-	-	-	-

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## **ROBUST AND WIDE SPECTRUM RNA SILENCING MEDIATED RESISTANCE TO *PLUM POX VIRUS***

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*Plum pox virus* (PPV) is a quarantine virus that causes sharka, one of the most important diseases of stone fruits worldwide. Seven PPV strains have been characterized, of which PPV-D, -M and Rec are the most important from an agro-economical point of view. The best agricultural sustainable approach to prevent sharka disease consists in developing PPV-resistant plants. In this context we have shown that *Nicotiana benthamiana* plants transformed with PPV-M sequences (PPV-IsPaVe44), covering the 5'UTR region, P1 and HC-Pro genes and arranged to express self-complementary 'hairpin' RNAs, are immune to the homologous PPV-ISPave44. However, as the RNA silencing-mediated resistance operates in a sequence-specific fashion, transgenic plants harboring the four hairpin constructs were also challenged with viral isolates belonging to different PPV strains. All the transgenic plant lines were resistant to PPV-D, -M and Rec strains. Moreover, the transgenic plant line harboring the 5' UTR/P1 sequence was also resistant to isolates of PPV-EA and PPV-C strains that are distantly related to ISPave44. Since it's known that some abiotic - low temperature - and biotic stresses - mixed viral infection - could have a detrimental impact on RNA silencing-mediated viral resistances, 5' UTR/P1 plants were challenged with PPV under different conditions. Transgenic plants were resistant to PPV infection both at high (30°C) and low temperature (15°C). Furthermore, no susceptibility to PPV was observed in 5'UTR/P1 plants previously inoculated with *Potato virus Y* (PVY), *Cucumber mosaic virus* (CMV) or *Artichoke mottled crinkle virus* (AMCV) suggesting that prior virus-mediated expression of HCPro (PVY), 2b (CMV) and P19 (AMCV) RNA silencing suppressors was not able to defeat PPV resistance. The overall data suggest that the 5' UTR/P1 hairpin construct can be profitably used to confer resistance to the sharka disease in *Prunus* species.

## **PRODUCTION AND *IN VITRO* ASSESSMENT OF TRANSGENIC PLUMS FOR THE RESISTANCE TO *PLUM POX VIRUS***

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*Plum pox virus* (PPV) is the quarantine pathogen causing sharka, the most devastating disease of stone fruits. Genetic engineering represents an interesting tool to obtain elite cultivars resistant to PPV. However, the containment of transgenic plants and PPV is an important aspect to be considered during the plant evaluation process. Here, we report the production of transgenic plum clones together with their *in vitro* evaluation for PPV resistance. Hypocotyl slices of mature plum seeds, cv Stanley, were transformed by *Agrobacterium tumefaciens* with a PPV-M derived hairpin construct (h-UTR/) that showed high performance in model plant. Two transgenic clones, St24 and St28, were obtained. A protocol to establish *in vitro* culture, proliferation and maintenance of PPV-D infected GF305 peach [*Prunus persica* L.(Batsch)] and virus-free microcuttings was developed. Micropropagated shoots of the transgenic clones were *in vitro* micro-grafted onto PPV-D infected GF305 rootstock. After the permanent union between the grafted tissues the transgenic scions were analysed by ImmunoCapture-RT-PCR (IC-RT-PCR) for PPV-Detection. More than 97% of ST24 and 73% of ST28 tested plants were resistant to the heterologous strain of PPV. Transgene-specific siRNAs were detected in both resistant clones, indicating that post-transcriptional gene silencing underlies the mechanism of resistance. These results confirm the role of the genetic engineering for the development of plum clones highly resistant to PPV and offer a quick and contained infection test procedure of practical interest.

## USE OF HORTICULTURAL MINERAL OIL TREATMENTS FOR THE CONTROL OF DIFFERENT *PLUM POX VIRUS* ISOLATES IN NURSERY BLOCKS

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The effect of horticultural mineral oil (HMO) treatments (Sunspray Ultrafine at 1%) on the natural spread of *Plum pox virus* (PPV), was evaluated in three experimental nursery plots of Nemaguard and Mariana GF8-1 *Prunus* rootstocks. The experimental plots were established under high natural PPV-inoculum pressure of different types in Plovdiv (Bulgaria)/PPV-M and -Rec, Bistrita (Romania)/PPV-D and -Rec and Llíria (Spain)/PPV-D. Treatments were applied weekly applied during the vegetative period from springtime to fall (in summertime the treatments were interrupted). Nursery plants were yearly analysed by MagicDAS-ELISA (Plant Print Diagnostics) based on the monoclonal antibody 5B-IVIA. The aphid population of each experimental nursery plot was monitored by Moericke yellow traps and by the sticky-shoot method. Springtime was the period when the maximum peak of aphid population was registered in the three experimental nursery plots. PPV incidence was significantly reduced ( $p < 0.05$ ) in all the experimental plots and in both assayed rootstocks, although the HMO treatments did not avoid PPV infection, probably due to the high PPV prevalence present in the neighbouring area of the experimental nursery blocks. This result shows that HMO treatments could be used as a possible environmental friendly control strategy, in different ecological areas, to reduce PPV incidence in *Prunus* nurseries regardless the PPV isolate involved, due to the physical action of the film of oil that covered the surface of the treated plants.

## THE PRESENT STATUS OF COMMERCIALIZED AND DEVELOPED BIOTECH (GM) CROPS, RESULTS OF EVALUATION OF PLUM HONEYSWEET FOR RESISTANCE TO PLANT VIRUSES IN THE CZECH REPUBLIC

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### Abstract

Commercialization of biotechnological (GM) crops started in 1995. A significant increase of 9 million hectares planted Biotech crops per year was realized in 1996-2009. In the years 2010-2011 it was already 12 million hectares (8%). 16.7 million farmers in 29 countries planted 160 million hectares of Biotech crops in 2011. Developing countries grew close to 50% of global Biotech crops at present. The U.S.A. is the lead producer of Biotech crops with 69 million hectares, 43% of global. GM soybean remains dominant crop, followed by Bt maize, cotton, and canola. Golden Rice is advancing towards the completion of its regulatory requirements. GM crops are contributing to the reduction of CO<sub>2</sub> emissions. Not only field crops, but also horticultural transgenic crops are developed and commercialized. The impressive progress is in Biotech vegetable projects which include tomato, potato, cabbage, brassica, cauliflower, bean, sweet pepper, chili, zucchini, squash, eggplant, cucumber, carrot, and sweet corn. Genetic engineering has the potential to revolutionize fruit tree breeding.. The development of transgenic fruit cultivars is in progress. Papaya resistant to *Papaya mosaic virus* is grown in the U.S.A. and China. Biotech grapevine resistant to viral, bacterial, fungal disease with abiotic stress tolerance and health benefits are grown in South Africa. Biotech banana, apple, pear, and strawberry cultivars are under the development. A result of over the past 20 years an international research is development of 'HoneySweet' plum highly resistant to PPV. GM plum 'HoneySweet' resistant to *Plum pox virus* (PPV) was deregulated in U.S.A. in 2010. Plum clone C-5 (cv. 'HoneySweet' at present) was ten years evaluated in the Czech Republic for resistance to PPV, PDV, and ACLSV under the high and permanent infection pressure of PPV and combinations with PDV, and ACLSV. 'HoneySweet' plum proved to be highly resistant in leaves and fruits to PPV. The mixed infection of PPV-With PDV and/or ACLSV has practically no influence on the quantity and

quality of HoneySweet fruits. Fruits from 'HoneySweet' trees graft-inoculated with PPV and combinations with PDV, and ACLSV are even better quality than fruits from healthy control trees of plum 'Stanley'.

## Introduction

Biotechnologies and biotechnological (Biotech), syn. genetically modified (GM) crops are the only possibility for mankind to survive under the conditions when the world population is growing all the time. Biotech crops have an enormous potential for contributing to the Millennium Development Goals (MDG) of reducing poverty by 50% by 2015. Three billion of the estimated world population for 2050 means the one third of the total population will suffer from hunger if GM crops are not grown.

Research in genetic engineering started in late seventies (Colwell *et al.*, 1985). The first genetically modified (GM), syn. biotechnological (Biotech) crops were commercialized in 1995 (cotton, by Monsanto; potato, by Syngenta). 1.7 million hectares of Biotech crops (cotton, corn, potato) were planted in 1996 already (USDA-APHIS Biotechnology Permits Database, available at <http://www.nbiap.vt.edu/> (March 2001). Increase in hectareage to 160 million hectares in 2011 (Fig. 1) makes Biotech crops the fastest adopted crop technology in the history of agriculture. The International Service for the Acquisition of Agri-biotech Applications (ISAAA) is every year publishing 'Global status of commercialized Biotech/GM crops'. The last one was published as ISAAA Brief No. 43 in 2011 (James C., 2011). The objective of this Brief is to provide information and knowledge to the scientific community and society on Biotech/GM crops and its contribution to global food, feed, fiber and fuel security, to sustainable agriculture.

Of the 29 countries planting Biotech crops in 2011 (Fig. 2), 19 were developing countries and 10 were industrial countries. 60% of the world's population live in countries planting Biotech crops. The US is the lead producer of Biotech crops with 69 million hectares. The US is followed by Brazil with 30.3 mil. ha, Argentina (23.7 mil. ha), India (10.6 mil. ha), and Canada (10.4 mil. ha). Developing countries grew close to 50% of global Biotech crops. Another five countries, China, Paraguay, Pakistan, South Africa, and Uruguay each grew more than 1 million hectares. Biotech soybean remains with 75.4 million hectares (47% of global Biotech area) the dominant crop, followed by Biotech maize with 51 million hectares, Biotech cotton 24.7 mil. ha (Fig. 3), and Biotech canola 8.2 mil. ha.

The only 0.114507 million hectares of Biotech crops were planted in Europe. Six EU countries, Spain, Portugal, Czechia Republic, Poland, Slovakia, and Romania planted 114490 ha of Bt maize. Sweden (15) and Germany (2) planted 17 ha of Biotech starch potato cv. 'Amflora'. Spain as the first European country is with 97326 ha of Bt maize on the 17. place. In the Czech Republic the Bt maize was grown on 5091 ha in 2011.

Biotech crops are strongly contributing to food security. This was achieved from 1996 to 2010 (in five years) by: increasing crop production and value by 78 billion US dollars, providing a better environment.

1. saving 443 million kg a.i. of pesticides.
2. in 2010 alone reducing CO<sub>2</sub> emissions by 19 billion kg, equivalent to taking 9 million cars off the road.
3. saving 91 million hectares of land.
4. helping 15 million farmers, the poorest people in the world.
5. From the point of food security European policy is going against humanity in the world.

European policy in relation to GM crops is scandal. In October 2011, forty one leading Swedish biological scientists in a strongly-worded open letter to politicians and environmentalists asked to revise European legislation to allow society to benefit from Biotech crops. A contingent of scientists from the United Kingdom endorsed the Swedish petition. I would like to ask scientists present on the international conference in Roma 2012, to join Swedish and UK scientists.

### Materials and Methods

Plum (*Prunus domestica* L), clone C5 transformed with the Plum pox virus (PPV) coat protein (CP) was obtained by Scorza *et al.* (1994). Clone C5 (cv. 'HoneySweet' at present) was proved to be highly resistant to PPV under glasshouse conditions (Ravelonandro *et al.*, 1997). Field tests were conducted in Poland (Malinowski *et al.*, 1998), Romania (Ravelonandro *et al.*, 2002), Czech Republic (Polák *et al.*, 2005), and in Spain (Malinowski *et al.*, 2006). The all experiments confirmed the resistance of clone C5 to PPV infection.

A trial of high and permanent infection pressure of PPV-Rec alone and in combinations with *Prune dwarf virus* (PDV), and *Apple chlorotic leafspot virus* (ACLSV) was initiated in the Czech Republic and published (Polák *et al.*, 2008a; 2008b). Summarized results of ten years testing of plum clone C5 under the high and permanent infection pressure both from graft inoculation and natural aphid vectors are presented. The Biotech plum trees were evaluated during the years 2002-2011.

**Field trials, transgenic plum trees, inoculation of viruses:** Plum clone C5 buds were grafted onto virus-free rootstocks of St. Julien in 2002, and 55 trees of *P. domestica* clone C5/St. Julien were obtained. Eleven trees in each combination were inoculated with PPV-Rec, PPV-Rec + ACLSV, PPV-Rec + PDV, PPV-Rec + ACLSV + PDV, control non-inoculated trees were obtained and a plantation established (Fig. 4). PPV-Rec, ACLSV, and PDV infected buds were allowed to grow throughout the period of evaluation. The GM clone C5 part of each tree was under a very strong inoculum pressure.

**Evaluation of leaf and fruit symptoms, quality of fruits:** The all trees were evaluated every year from May to September for the presence of viral symptoms in leaves (2002-2011). Fruit symptoms were evaluated in July and August 2010 and 2011 before and at ripening. In 2010 and 2011 overall fruit uniformity, attractiveness, weight, length, width and fruit thickness, flesh thickness, fruit shape, skin colour, flesh colour, flesh firmness, flavour, flesh freeness, total soluble solids, total titratable acidity, stone size, weight and stone/flesh ratio, and dry weight of fruits harvested

from trees of clone C5 inoculated with PPV-Rec, and no-inoculated control trees of clone C5, and 'Stanley' were recorded.

**Serological detection of viruses:** ELISA testing of leaves was performed every year in June. Fruits were evaluated in August 2010 and 2011. Polyclonal antibodies raised against PPV, ACLSV, and PDV (Bioreba, Switzerland) were used in DAS-ELISA (Clark and Adams, 1977). Leaf samples were extracted in phosphate-buffered saline. The relative concentration of PPV-Rec was determined by semiquantitative DAS-ELISA in samples prepared from symptomatic leaves in June 2005 and 2007, and in samples prepared from fruits in August 2010 and 2011. The relative concentration of PPV protein was established by determining the lowest dilution of leaf or fruit sample with positive reaction in semi-quantitative DAS-ELISA (Albrechtová *et al.*, 1986).

**Detection of viruses by Reverse Transcription-Polymerase chain reaction (RT-PCR):** 100 mg of ground leaf or fruit tissues were used for total RNA extraction by using RNeasy Plant Mini Kit (QIAGEN GmbH, Hilden, Germany) according to the procedure recommended by the manufacturer. PPV-Rec was detected by RT-PCR using primer pair mD5/mM3 as described by Šubr *et al.* (2004). For PDV and ACLSV the primers as described by Jarošová and Kundu (2010) were used.

## Results and Discussion

No PPV symptoms appeared in the leaves of Biotech plum clone C5-cv. Regarding 'HoneySweet' trees in the first year after inoculation with PPV-Rec by graft inoculation. PPV symptoms appeared only in the leaves that emerged from infected buds (IB). Mild diffuse spots and rings appeared two years after inoculation in some basal leaves of cv. 'HoneySweet' trees inoculated with PPV-Rec, and in those inoculated with virus combinations PPV-Rec + ACLSV, PPV-Rec + PDV, and PPV-Rec + ACLSV + PDV (Polák *et al.*, 2005). PPV presence was confirmed by ELISA and RT-PCR. A reduction of symptoms was observed in the third year after virus inoculation. PPV symptoms were observed only in several basal leaves and symptoms were milder each year (Polák *et al.*, 2008a). Further reduction of PPV symptoms was observed during the vegetation period from June to September. No differences in the intensity of PPV leaf symptoms among different virus combinations were observed in the years 2003-2011. No symptoms of PDV and ACLSV appeared during the vegetative periods of 2003-2011. PDV was not detected by ELISA in GM parts of trees inoculated with PPV-Rec + PDV and PPV-Rec + PDV + ACLSV. The presence of PDV was inconclusive by RT-PCR. PDV was detected by ELISA and RT-PCR only in leaves growing from the IB. ACLSV was detected by ELISA and RT-PCR in leaves of transgenic parts of trees inoculated with PPV-Rec + ACLSV and PPV-Rec + PDV + ACLSV. No symptoms of PPV, PDV, and ACLSV appeared in the leaves of non-inoculated control trees of cv. 'HoneySweet' throughout the experiment. PPV, PDV, and ACLSV were not detected in control trees by DAS-ELISA and RT-PCR. The growth of control trees was more vigorous in comparison with trees inoculated with PPV, and combinations with PDV and ACLSV. The severe PPV symptoms which appeared first in 2003 in IB leaves growing from buds infected with PPV-Rec appeared again every year (2004-2011) with the same intensity.

'The relative concentration of PPV-Rec in symptomatic leaves of cv. 'HoneySweet' determined by semi-quantitative DAS-ELISA fluctuated from  $1.56 \times 10^{-2}$  to  $9.76 \times 10^{-4}$  in 2005, and from  $5.0 \times 10^{-1}$  to  $7.81 \times 10^{-3}$  in 2007. There were no significant differences in relative concentration of PPV among combinations of inoculated viruses. Pomological evaluation of external and internal characteristics of fruits harvested from non-graft-inoculated cv. 'HoneySweet' trees, cvs. 'Stanley', and 'Domáci švestka' trees, and from cv. HoneySweet' trees growing nine years under the high and permanent infection pressure of PPV-Rec, PPV-Rec + PDV, PPV-Rec + ACLSV, PPV-Rec + ACLSV + PDV demonstrated the high quality of 'HoneySweet' fruits. Two-year results indicate that the characteristics of 'HoneySweet' fruits harvested from control virus non-inoculated trees are well within the range of the characteristics of control cultivars 'Stanley' and 'Domáci švestka' and are of higher quality in some characteristics. Fruit harvested from 'HoneySweet' trees inoculated with PPV-Rec + ACLSV + PDV, PPV-Rec + PDV, PPV-Rec + ACLSV, and PPV-Rec were comparable with fruits from control healthy 'm 'HoneySweet' trees indicating that there was little, if any, effect of the virus inoculations on fruit quality of 'HoneySweet'.

'HoneySweet' plum trees resistant to PPV remained virus-free under natural aphid-vectored infection pressure throughout this ten-year study. Graft inoculated trees were exposed to a very high infection pressure with IB being allowed to reach the size of 20-30% of the supporting 'HoneySweet' tree (Fig. 5a, b). Under this high and permanent virus pressure 'HoneySweet' trees showed PPV symptoms and positive serological and molecular tests on some basal leaves only, and symptoms subsided during the growing season. ACLSV infection did not appear to affect PPV symptoms and PDV infection could not be detected in 'HoneySweet' throughout the course of the study despite graft inoculation.

The evaluations of fruit quality of graft inoculated and un-inoculated 'HoneySweet' trees maintained ten years under the high and permanent infection pressure of PPV, ACLSV, and PDV confirmed not only the high resistance of 'HoneySweet' to PPV, but also suggested that 'HoneySweet' fruits maintain their quality and healthful properties when exposed not only to 0PPV but also to ACLSV and PDV. Clone C5, resistant to PPV, was deregulated as plum 'HoneySweet' in the U.S.A. in 2010. The meeting of U.S. International Research team for 'Honey Sweet' plum in Prague, May 2011 decided to submit an application for deregulation of plum cv. 'HoneySweet' in European Union. Members of International team are scientists from USA, France, Spain, Poland, Romania, Bulgaria and the Czech Republic. The Czech Republic is represented by Ass. Prof. Jaroslav Polák, DrSc. and Dr. Jiban Kumar from Crop Research Institute in Prague. International team under the leadership of Dr. Ralph Scorza, U.S.A. and Dr. Michel Ravelonandro, France is setting up the application for deregulation of 'HoneySweet' in EU. Crop Research Institute, Prague, and the Czech Republic are supposed to submit the application to EFSA. J. Polák is coordinating and setting up results of research on plum 'HoneySweet' in Europe. The growing of GM plum is supported by public, the Fruit Growers Union, Nursery men's Union, by Czech politicians. The growing of plum 'HoneySweet' in the Czech Republic will be principal contribution not only for fruit growers, but also for producers of plum brandy. It is unique opportunity to establish PPV free orchards and to grow high quality fruits.

The future is to grow of high quality and productive crops resistant to plant diseases and pests, tolerant to herbicides, to ensure food security and to prevent hunger of people in the world. Genetic engineering will make possible to obtain high quality crop cultivars today very susceptible to diseases, highly resistant in the future, e.g. plum 'Domáci švestka' highly resistant to PPV.

#### Acknowledgements

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## CONIFER EXTRACTS AS REPELLENTS FOR *CACOPSYLLA PRUNI* AND THEIR EFFECTS ON THE SPREAD OF ESFY IN APRICOT

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### Abstract

Conifers act as shelter plants for *Cacopsylla pruni*, vectors of European stone fruit yellows (ESFY), during autumn-winter time. The abandonment of conifers in spring is reported as a crucial point in the life cycle of *C. pruni*; a repellent mechanism induced by conifers on the insect has been hypothesized to underlie this behavior. In an experimental trial established under semi-field conditions in an insect-proof tunnel, a total of 90 one-year-old apricots cv. Reale d'Imola were used. Plants were divided into three types: untreated, treated with systemic insecticide, treated with *Picea abies* extract. Starting from two days after treatment, a total of 500 insects collected in areas with high ESFY disease pressure were released in the tunnel. Distribution of the released insects on the test plants was visually recorded three times over the following twenty days. Results showed a greater presence of *C. pruni* on untreated apricots than on plants treated with insecticide or with conifer extract. These differences were confirmed and emphasized also by the monitoring of the new generation insects on the apricot plants of the three types. In order to estimate ESFY diffusion in this case study, real-time PCR analyses were carried out on samples of the apricot plants at the end of the vegetative season. Molecular analyses highlighted a reduced diffusion of ESFY in the insecticide and conifer-extract treated plants, suggesting a repellent mechanism exercised by the *P. abies* extract on *C. pruni*. In fact in the semi-field conditions adopted, apricot colonization by *C. pruni* was reduced and ESFY diffusion was comparable between insecticide-treated plants and conifer-extract-treated plants. Characterization of the *P. abies* extract is undergoing in order to identify compounds involved in the repellent mechanism.

### Introduction

European stone fruit yellows (ESFY) is a phytoplasma disease caused by '*Candidatus Phytoplasma prunorum*' ('*Ca. P. prunorum*'), and is one of the more important diseases that affect *Prunus spp.* across a number of European countries. The incidence of the disease differs in each country and is related to the *Prunus* species and the environmental conditions present. '*Ca. P. prunorum*' belongs to subgroup 16Sr

X-B of the 'Apple proliferation' taxonomic group (Seemüller and Schneider., 2004). The host range of '*Ca. P. prunorum*' is quite large and includes wild and cultivated *Prunus* species. Among cultivated species, apricot (*P. armeniaca* L.), Japanese plum (*P. salicina* Lindl) and peach (*P. persica* L. Batsch) are the most damaged crops observed under field conditions. In nature, ESFY is spread by *Cacopsylla pruni* (Scopoli) (Carraro *et al.*, 1998), which is common in Europe at various latitudes, from southern Finland to Portugal and to the Caucasus and Siberia (Ossiannilsson, 1992). This psyllid species, like *C. melanoneura* and *C. picta*, which are vectors of Apple proliferation (AP), is univoltine and overwinters as adults on conifers. Reimmigrant adults, at the end of winter move to *Prunus* species for coupling and oviposition. The mechanisms that regulate reimmigration and in general the orientation and host finding by these phloem-feeding insects is not completely clear (Weintraub and Beanland 2006). In the AP/*C. melanoneura* and *C. picta* system the olfactory response of the two species indicated that plant kairomones may play a role in host-finding mechanisms (Gross and Mekonen 2005). Mayer and co-workers (2008) demonstrated also that phytoplasma infection changes the volatile emission profile and behaviour of insect vectors. It is still not understood if conifers play an active role in psyllid abandonment of the trees at the end of winter.

The aims of this study were to better understand the biological mechanisms of interaction between *C. pruni* and their hosts and to find environmentally friendly methods for control of the *C. pruni* population and the spread of ESFY in apricot orchards.

## Materials and Methods

The field experimental design consisted of 90 apricot plants (cv Reale d'Imola) grown inside a greenhouse and arranged in two 4X1 m rows. In both 2011 and 2012 the experimental design consisted of three groups of 30 plants each: a) distilled water treated; b) treated with Imidacloprid insecticide (02g active ingredient/l); c) treated with *Picea abies* extract. For the conifer extract preparation, shoots of *Picea abies* were collected just before use and chipped in a grain mill; chips were rinsed in hot water (100g/l) for 15 minutes, the extract was filtered through gauze and sprayed on test plants at the same volume used for the insecticide application. The treatment schedule included two insecticide applications for group b) (phenological stages BBCH09 and BBCH69) and three application application on groups a) and c) (BBCH09, BBCH69 and BBCH71). *C. pruni* reimmigrant adults were captured in an apricot orchard located in a high ESFY disease pressure area in the Friuli Venezia Giulia region (N.E. Italy). The method of capture was to place a white cloth stretched over a tray under infested tree branches as they were beaten and to then collect the insects with an aspirator. Immediately after capture insects were randomly released under the experimental tunnel. Nucleic acids from individuals of *C. pruni* were extracted according to Doyle and Doyle (1990) and individually tested by nested-PCR using AceFf1/AceFr1 and AceFf2/AceFr2 primers to ascertain the presence of '*Ca P. prunorum*' (Danet *et al.*, 2008). Visual inspections of test plants for the presence and distribution of insects were made one, two, and three weeks after the release. In

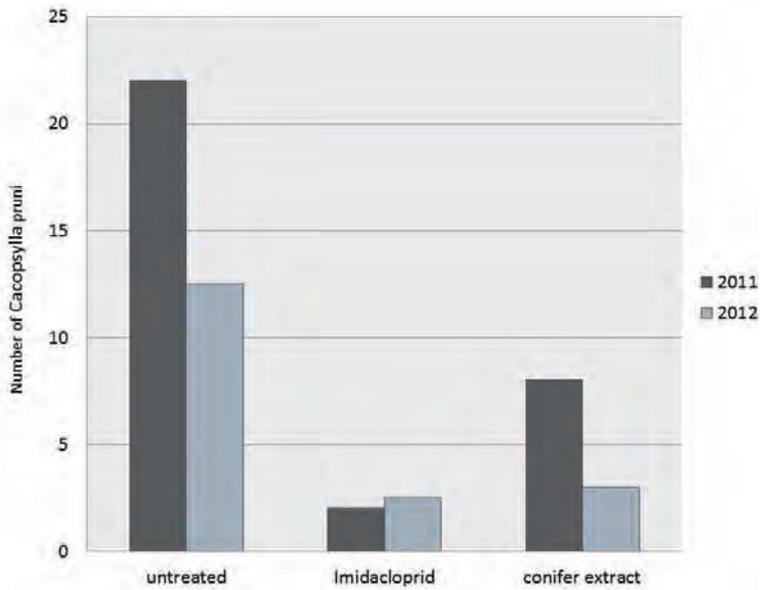


Fig. 1 - The numbers of *Cacopsylla pruni* on *Prunus armeniaca* sprayed with Imidacloprid, conifer extract or untreated in 2011 and 2012.

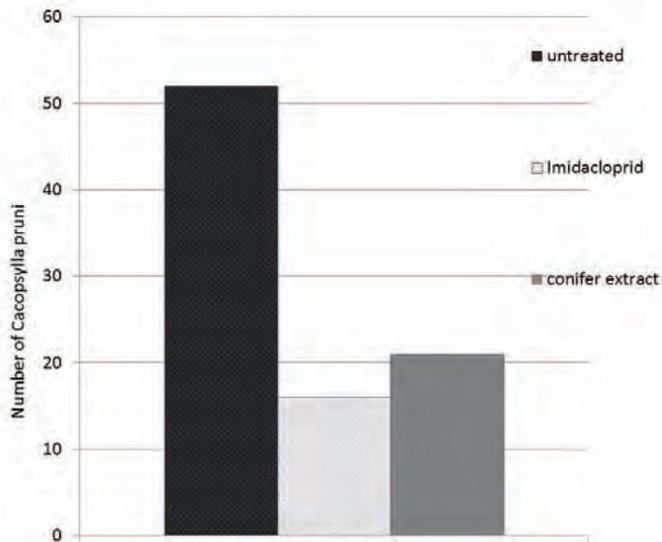


Fig. 2 - The numbers of new generation *Cacopsylla pruni* on *Prunus armeniaca* sprayed with Imidacloprid, conifer extract or untreated (2011 data).

September 2011 leaves from apricot plants were collected for '*Ca. P. prunorum*' detection by real-time PCR (SYBR<sup>®</sup> Green I) using rpLNS2f / rpLNS2r primers (Martini *et al.*, 2007).

## Discussion

The results obtained in the present work show a repellent action of conifer extracts on colonization by reimmigrant *C. pruni*. This suggests that there is a repellent effect associated with the water extractable compounds of *Picea abies*. In addition, plant colonization by insects of the new generation was proportional to the numbers of reimmigrant *C. pruni*, and this suggested a low mobility of the new generation until they left the primary host in the search of conifers. The spread of ESFY was correlated with the presence of insect vectors, with a higher incidence of the disease in the untreated control. The results obtained highlight the risk associated with diseases such as ESFY, which can assume an epidemic trend over short periods of time if vector control is not implemented. At the same time, while insecticide treatments ensure a strong reduction in the population of the vector they are not able to eliminate the spread of ESFY. In the future it would be interesting to evaluate whether the results obtained in the greenhouse are repeatable under field conditions. In particular, treatments with conifer extracts may be of value in organic farming where most of the available insecticides are not persistent enough to cover the *C. pruni* reimmigration period and require multiple applications. At the same time, conifer extracts may play a role as an insecticide adjuvant in conventional farming to improve the effectiveness of conventional insecticides.

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## USE OF MICROPROPAGATED MALUS TO STUDY LATENT APPLE VIRUSES

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### Abstract

Latent apple viruses like *Apple stem grooving virus* (ASGV, *Capillovirus*) and *Apple stem pitting virus* (ASPV, *Foveavirus*) were maintained in *Malus x domestica* cultivars micropropagated *in vitro*. Analyses of the genetic variability among different isolates of both viruses exhibited a high heterogeneity in each group of virus. However, micropropagation proved to be a valuable tool to stably maintain a defined isolate of both ASGV and ASPV over a long-term period. This is a prerequisite for further studies on the interaction of both viruses with ‘*Candidatus Phytoplasma mali*’, the causal agent of apple proliferation disease, as well as the analysis of the necrotic reaction shown by *Malus sieboldii* upon infection with latent apple viruses. The analysis of this necrotic reaction of *M. sieboldii* has become of major interest since *M. sieboldii* and its hybrids confer resistance to apple proliferation (AP) disease and have been used in a recent breeding program aimed at developing AP-resistant rootstocks of agronomic value. *In vitro* grafting was successfully applied to transmit both viruses to healthy plants or to establish defined co-infections of different viruses and ‘*Ca. P. mali*’ isolates. The virus titer in the inoculated plants was determined by qRT-PCR analysis.

### Introduction

*Apple stem grooving virus* (ASGV, *Capillovirus*) and *Apple stem pitting virus* (ASPV, *Foveavirus*) are wide spread throughout all apple growing regions. Although these viruses are considered latent on most *Malus x domestica* cultivars, they have been associated with topworking disease and necrosis in *Malus sieboldii* derived cultivars (Yanase, 1974, 1981). *M. sieboldii* cultivars confer resistance to ‘*Candidatus Phytoplasma mali*’ (Seemüller *et al.* 2008), and this resistance was successfully exploited in crossings with *M. x domestica* rootstock cultivars to breed commercially viable apple proliferation resistant rootstocks (Jarausch *et al.*, 2011). Some of the *M. sieboldii* hybrids, however, showed a high susceptibility to latent apple viruses such as ASGV and ASPV and resulted in necrosis or death of the plant (Seemüller *et al.* 2008; Ciccotti *et al.* 2011).

Both ASGV and ASPV are from the family *Betaflexiviridae* and belong to the genera *Capillovirus* and *Foveavirus*, respectively (Martelli *et al.* 2007). ASGV and

ASPV both have a single-stranded positive sense genomic RNA, encapsulated in flexuous viral particles. The ASGV genome encodes a replicase, movement and coat protein (Jelkmann, 1994; Koganezava and Yanase, 1990; Ohira *et al.* 1995; Yoshikawa *et al.* 1992). The ASPV genome encompasses 5 open reading frames, encoding for replicase, the triple gene block, and the coat protein. ASPV shows a high degree of variability, the coat protein gene being one of the highly variable areas (Nemchinov *et al.* 1998; Schwarz *et al.* 1998; Yoshikawa *et al.* 2001). ASGV has a more conserved genome compared to ASPV, with the exception of two highly variable areas, variable region 1 (532-570aa) and variable region 2 (1 583-1 868aa) (Liebenberg *et al.* in press, Tatineni *et al.* 2009).

The study of apple viruses and their symptom expression *in vivo*, either in field or greenhouse trials, is time consuming, and the analysis of symptom expression is further hampered by external factors like temperature, ground nutrients and secondary pathogens. To study the effect of the one virus isolate on the symptom expression of *M. sieboldii* derived rootstocks, a system is necessary where all other influencing factors can be held constant. Among these, a genomic stable virus isolate that can be easily transmitted and maintained is needed. For this, the use of micropropagated plants is a valuable tool for both molecular and biological studies. A short overview of the potential of this approach is given in the present work.

### Materials and Methods

Healthy *Malus* shoot cultures were propagated in a growth chamber on modified Murashige & Skoog medium as described (Ciccotti *et al.* 2008; Jarausch *et al.* 1996, 1999). The plantlets were subcultured every 6 to 8 weeks. ASGV and ASPV infected material was sampled from infected field trees and *in vitro* culture lines were established according to a protocol described by Ciccotti *et al.* (2008) (Tab. 1).

**Tab. 1** - Virus isolates established from infected material through culture

Isolate	Virus	Origin
AC	ASGV	Germany
CO2	ASGV	Canada <sup>a</sup>
CO3	ASGV	Canada <sup>a</sup>
CO4	ASGV	Canada <sup>a</sup>
CO5	ASGV	Canada <sup>a</sup>
CO6	ASGV	Canada <sup>a</sup>
CO7	ASGV	Canada <sup>a</sup>
GO7	ASGV	Canada <sup>a</sup>
Stutt	ASGV	Germany <sup>b</sup>
LFP	ASPV	Germany
PMZ	ASPV	Germany
PMH	ASPV	Germany

ASGV infected plant material were obtained from a) Dr. Mike Bernardy, Pacific Agri-Food Research 'Candidatus Phytoplasma mali' infected *in vitro* cultures were those described by Bisognin *et al.* (2008).

The infected culture lines were used as grafting material for *in vitro* graft inoculation experiments (Jarausch *et al.* 1999) on healthy *Malus* plantlets to produce single and multiple pathogen culture lines. Healthy genotypes were *M. x domestica* cv. RubINETTE, *M. sieboldii* and *M. sieboldii* derived rootstocks (Ciccotti *et al.* 2008). The graft contact was maintained for 4 weeks and then, graft tips were removed. Inoculated genotypes were further subcultured for 6-8 weeks and then tested for infection by PCR.

Total nucleic acids from the inoculated rootstocks were extracted using the CTAB method described in Jarausch *et al.* (2011) and tested with RT-PCR using pathogen specific primers for ASGV and ASPV (Massart *et al.* 2008) to confirm the infection. ASGV and ASPV were further tested with qRT-PCR for relative virus titer analysis, using the Superscript III Platinum SYBR green one-step qRT-PCR kit with the primers ASGV\_uni-1685-F : GAGTCAAATCCGACGAAAGC and ASGV\_Centre, Agriculture & Agri-Food Canada, Summerland, British Columbia V0H 1Z0, and b) Michael Petruschke, Landwirtschaftliches Technologiezentrum Augustenberg – Außenstelle Stuttgart, Rötestrasse 16a, 70197 Stuttgart AC-1876-R: GAACATCAATGCCGAGGT for ASGV and the primers qASPVfor: CTTACAGTGACGCGCCGCA and qASPVrev: GGGAGCCCCAACATTGCCACC for ASPV.

An area of high variability was selected for each virus to amplify and sequence the variable area 1 of ASGV and the coat protein of ASPV, according to standard procedures. BioEdit (Ver. 7.0.4) (Hall, 1999) was used to perform sequence editing and compilation.

## Results and Discussion

*In vitro* shoot culture lines infected with nine ASGV isolates from Germany and Canada and three ASPV isolates from Germany were successfully established by culture initiation. ASGV isolates AC and Stutt and ASPV isolate LFP were *in vitro* grafted on different healthy *Malus* genotypes to produce single infected culture lines. The defined virus isolates were successfully maintained on micropropagated *Malus x domestica* cv. RubINETTE for up to 10 years. Virus-infected cultures showed no growth alteration compared to healthy controls whereas 'Ca. P. mali'-infected cultures of *M. x domestica* exhibit severe symptoms and stunting (Jarausch *et al.*, 1996; Bisognin *et al.*, 2008).

The genetic stability of the virus isolates was analysed on the most variable areas in the genomes of ASGV and ASPV, respectively. The variable area 1 of ASGV-AC was sequenced 3 times (2008, 2009, 2010) over a period of 3 years and the ASPV-LFP coat protein twice (2010, 2012) over a period of 2 years. Sequence comparisons for both the ASGV variable area 1 and the ASPV coat protein show an identity of 100% for all sequences obtained, respectively. These results prove that we were able

to stably maintain a defined isolate in *in vitro* plantlets.

*In vitro* grafting as described by Jarausch *et al.* (1999) was applied to produce defined virus-virus combinations (e.g. ASGV-AC plus ASPV-LFP) or defined virus-phytoplasma co-infections on *Malus x domestica* cultivars. In addition, *in vitro* grafting was also used to study the hypersensitive reaction of *M. sieboldii* and its hybrids to apple viruses. Transmission rates varied among virus and rootstock cultivar, with ASGV having a transmission rate of between 6 and 50% and ASPV between 14 and 80% depending on the hypersensitivity of the culture, with *Malus x domestica* cultivars showing the highest transmission rates and the *M. sieboldii* / *M. sieboldii*-derived rootstocks the lowest.

Hypersensitivity analysis showed necrotic symptoms in 38% of ASGV *in vitro* graftings on *M. sieboldii*, 0% on *M. x domestica* cv. RubINETTE and between 0% and 17% on *M. sieboldii* derived rootstocks. ASPV produced necrotic symptoms on 28% of *M. sieboldii* rootstocks, 0% on *M. x domestica* cv. RubINETTE and between 0% and 10% on *M. sieboldii* derived rootstocks (Liebenberg *et al.* 2010).

One-step qRT-PCR assays were developed to measure the concentration of ASGV and ASPV, respectively, in the inoculated plantlets. One example for an application of this technique in combination with *in vitro* plantlets is the analysis of the replication efficiencies of the different ASGV isolates. For this, repetitions of singly infected shoots of the same *Malus x domestica* cultivar were analysed after a defined subculture interval. The results showed that all ASGV isolates had similar replication efficiencies, with the ASGV-AC isolate having the highest titer per plant cell relative to the other eight ASGV isolates (data not shown).

## Conclusion

In this study we developed a system to study the effects of ASGV and or ASPV on *Malus*. Homogenous culture lines were established through *in vitro* grafting of the biologically important isolates on *M. sieboldii*, *Malus x domestica* and *M. sieboldii* derived rootstocks. Both ASGV and ASPV were easily transmitted to an array of *Malus* genotypes to produce virus-virus and virus-phytoplasma combinations. We have shown that it is possible to reproduce field symptoms of necrosis *in vitro*. We produced a system that gives easy access to a genomically stable, replicating virus.

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## PRELIMINARY RESULTS ON STUDIES OF RESISTANCE TO *PLUM POX VIRUS-D* IN *PRUNUS* IN ARGENTINA

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### Abstract

Sharka, caused by *Plum pox virus* (PPV), is the most detrimental viral disease of stone fruit in the world. The use of resistant cultivars appears to be the only effective method to control the disease. In Argentina, PPV was detected in plum and apricot in November 2004. In this study, the ability of two peach cultivars (*P. persica* cv. 'Pavía Catherina' and 'Loadel') was evaluated, by artificial inoculation to allow for the PPV strain D isolated through their vascular tissues to move a long distance. The presence of PPV symptoms on leaves of GF 305 rootstocks, cultivars and GF 305 scions on the top, was evaluated during one cycle of study. DASI-ELISA and AC-RT-PCR were performed on each part of the studied plants. The genotypes studied allowed the virus movement through their vascular system in both directions in the first cycle of study. No PPV symptoms were observed on leaves of either of the genotypes, although they were positives were found by ELISA and PCR. GF 305 always showed clear symptoms and gave positives reactions in the performed tests. These results are preliminary; more growing cycles are needed to determine the tolerance of 'Pavía Catherina' and 'Loadel' peach cultivars to PPV-D Argentinean isolate.

### Introduction

*Plum pox virus* (PPV) is the causal agent of sharka disease, one of the most dangerous diseases of stone fruits. PPV is present in many areas of the world including Europe, Asia, North Africa and America (Nemeth, 1986; Mazyad *et al.*, 1992, Herrera, 1994, Bhardwaj *et al.*, 1995, Levy *et al.*, 2000, Thompson *et al.*, 2001, Navratil *et al.*, 2006, Fujiwara *et al.*, 2011). In Argentina, PPV was first detected on plum (*Prunus salicina* cv. 'Red Beaut') and apricot (*P. armeniaca* cv. 'Bulida') in San Juan state, in November 2004 (Dal Zotto, *et al.*, 2006). Since 2005, the Argentinean phytosanitary service (Servicio Nacional de Sanidad y Calidad Agroalimentaria-SENASA) has required nurseries to analyze stone fruit propagating material in order to avoid virus dissemination. After seven years of surveys and the elimination of infected material, a very low incidence (0.02 %) of the virus has been found. To date, only PPV-D type strains have been reported with none of the detections being found in peach (*P. persica*) (Dal Zotto, *et al.*, 2006; Rossini *et al.*, 2012).

The virus is difficult to control as it is transmitted in a non-persistent manner by aphids, is distributed unevenly throughout the plant, and occurs in low concentration. The use of resistant cultivars appears to be the only effective method to control the disease. (Dicenta *et al.*, 2000).

The method traditionally used to evaluate resistance to PPV has been the observation of symptom development on leaves in controlled greenhouse conditions (Dicenta *et al.*, 2000; Martinez-Gomez *et al.*, 2000). The results obtained have often been contradictory due to the inconsistent nature of viral infections (Martinez-Gomez and Dicenta, 2001). The study of the ability of a genotype to allow long-distance movement of the virus through its vascular vessels has been proposed as a method to evaluate resistance in herbaceous and woody species (Wang *et al.*, 1999; Sáenz *et al.*, 2002; Dicenta *et al.*, 2003; Rubio *et al.*, 2008). Here we describe our initial attempts to identify resistance to PPV\_D in two genotypes of peach from Argentina by examining long distance movement of the virus through the vascular tissue.

### Materials and Methods

**Plant material:** Seedlings of ‘GF 305’ peach, grown from seeds which originated from virus-tested mother plants, were used as both a rootstock and a susceptible control genotype. Two peach genotypes ‘Pavía Catherina’ and ‘Loadel’ were evaluated for resistance.

**PPV isolate:** The isolate used in this project was a PPV-D type isolate collected from Argentinean infected plum trees and maintained on ‘GF 305’ peach.

**Experimental design:** Two different plant models were constructed, as cited by Rubio *et al.*, 2008, to evaluate the ability of the studied genotypes to permit the movement of the PPV through their vascular system (Fig. 1). Five replications per model of each genotype under evaluation were studied during one cycle of growth under controlled

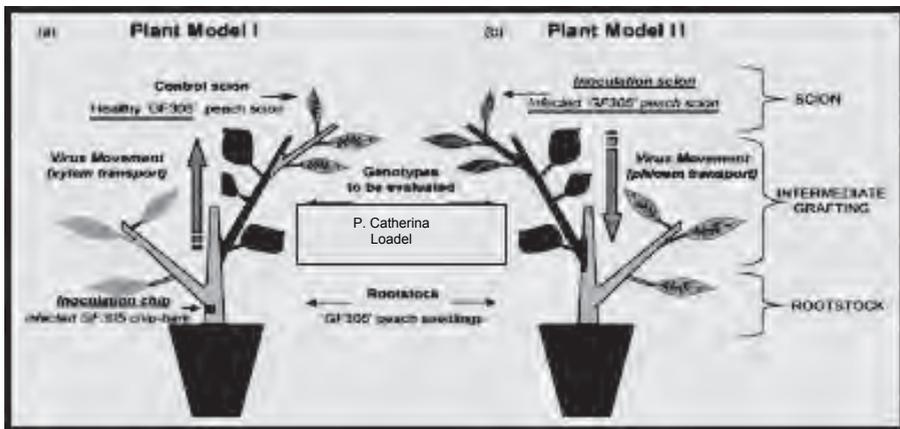


Fig. 1- Plant models assayed (from Rubio *et al.*, 2008).

insect-proof greenhouse conditions. One plant of each cultivar was left without virus inoculation as a negative control. One month after grafting PPV-infected 'GF 305' plants with the genotypes, the plants were subjected to an artificial rest period in a dark cold chamber at 4°C for two months. After this period the plants were transferred to the greenhouse, forced to sprout, and during the second growing season, when the genotype developed, an upper 'GF 305' bud was grafted (Fig. 1).

**PPV-Detection:** The presence/absence of symptoms in leaves of 'GF 305' and cultivars ('P. Catherina' and 'Loadel') was recorded each week from leaf emergence to leaf fall. At the end of spring, leaf samples were collected from the peach genotypes and from 'GF 305' and tested for the presence of PPV-D by DASI- ELISA (Cambra *et al.*, 1994) and AC-RT-PCR (Wetzel *et al.*, 1991).

## Results and Discussion

The results obtained in the evaluation of sharka resistance of the two peach genotypes studied are shown in Tab. 1. No PPV symptoms were observed on leaves of either of the cultivars ('P. Catherina' and 'Loadel') in Models I and II respectively, although PPV-Was detected in these tissues by ELISA and PCR. 'GF305' always showed clear PPV symptoms (Fig 2) and gave positive reactions in detection assays, showing the high susceptibility of this genotype to PPV , as described previously by several authors (Martínez-Gómez and Dicenta, 2000; Rubio *et al.*, 2008).

PPV-Was detected in 'GF 305' leaves at the upper part of the plant in both genotypes studied in Model I (inoculated at the bottom) (Tab. 1) indicating that both genotypes allowed virus movement through the plant xylem. In Model II (inoculated at the top), PPV virus was detected in the lower 'GF 305' leaves by ELISA, PCR and symptom expression, showing a movement through the phloem (Tab. 1)

The genotypes studied allowed virus movement through their vascular system in both directions regardless of the presence of symptoms, supporting the theory that the ability to allow, or not allow, the movement of the virus thorough the plant is a more accurate and stable trait to evaluate resistance/susceptibility (Rubio *et al.*, 2008).

The results presented in this work are preliminary and additional growing cycles are needed to determine the tolerance of 'P. Catherina' and 'Loadel' peach cultivars to PPV-D Argentinean isolate.

Tab. 1- PPV-D detection by symptom expression, ELISA and RT-PCR

<b>Model I (xylem transport)</b>	<b>Symptoms</b>	<b>ELISA</b>	<b>RT-PCR</b>
GF 305 bottom part (PPV inoculated)	+	+	+
Pavia Catherina	-	+	-
GF 305 upper part	+	+	+
Negative control	-	-	-
GF 305 bottom part (PPV inoculated)	+	+	+
Loadel	-	+	+
GF 305 upper part	+	+	-
Negative control	-	-	-
<b>Model II (phloem transport)</b>			
GF 305 bottom part	+	+	+
Pavia Catherina	-	+	+
GF 305 upper part (PPV inoculated)	+	+	+
Negative control	-	-	-
GF 305 bottom part	+	+	+
Loadel	-	+	+
GF 305 upper part (PPV inoculated)	+	+	+
Negative control	-	-	-

+ = ELISA, RT-PCR positive results or symptom expression  
 - = ELISA, RT-PCR negative results or not symptom expression

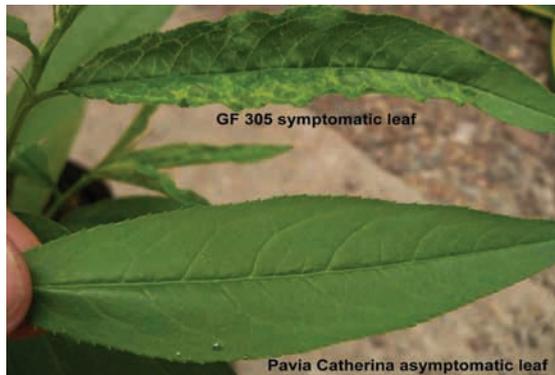


Fig. 2 - Sharka symptomatic ‘GF 305’ leaves and asymptomatic ‘P. Catherina’ leaves.

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## MONITORING OF FRUIT TREE PHYTOPLASMA VECTORS IN AN OVERWINTERING SITE

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Psyllids – vectors of fruit tree phytoplasmas – were studied in an overwintering site, locality Olšany (south Moravia, Czech Republic) between 2006-2010. The population dynamics of *Cacopsylla pruni* ('*Candidatus* Phytoplasma prunorum' vector), *C. picta* and *C. melanoneura* (both '*Candidatus* phytoplasma mali' vectors) were monitored and the incidence of phytoplasma positive individuals was determined by PCR/RFLP analysis. Using a sweeping net on a long stick a total of 803 individuals of the above mentioned vectors were collected. It was confirmed that conifers (*Picea abies* being the dominant species in the area) growing in the investigated highland site of central Moravia harbour *C. pruni*, *C. picta* and *C. melanoneura* during the winter season. Knowledge regarding the seasonal migration of the three studied psyllid species was increased. The sum of the captured individuals varied from year to year depending on population density, seasonal migration of psyllids and their activity, and weather conditions. *C. picta* was the least frequently captured of the three phytoplasma vectors studied. The presence of phytoplasma positive individuals reached 22% for *C. pruni* ('*Ca. P. prunorum*'), 15% for *C. picta*, and 14% for *C. melanoneura* (both '*Ca. P. mali*').

## RECOVERY PHENOMENA IN APRICOT TREES CV. BERGERON INFECTED BY EUROPEAN STONE FRUIT YELLOWS IN THE PROVINCE OF TRENTO (ITALY)

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### Abstract

Recovery phenomena have been intensively studied for apple proliferation and grapevine yellows. Several recent findings in apricot orchards in Friuli-Venezia Giulia indicate the importance of this strategy also for the control of European Stone Fruit Yellows (ESFY) infections, caused by '*Candidatus Phytoplasma prunorum*'. The aim of this work was to investigate the occurrence of recovery in 2 experimental orchards of apricot cv. Bergeron on 'Wavit', located in the province of Trento, where the disease has been spreading since 2000. This included visual inspections for typical symptoms and a diagnostic method based on a Spot Real-Time Reverse Transcription PCR assay. The results obtained showed that there were three different groups of plants infected by ESFY: many plants with typical symptoms, some recovered and some symptomless infected plants. To date no interesting plant genotypes showing resistance to ESFY are available, moreover most of the insecticide applications have no efficacy in controlling the vector than the disease. The use of both pathogen-free propagation material and resorting to natural recovery could be adopted as an integrated approach for the control of this phytoplasma disease. For these reasons the research will continue with the aim of quantifying the concentration of the pathogen in the recovered plants.

### Introduction

'*Candidatus Phytoplasma prunorum*' is associated with the quarantine phytoplasma European stone fruit yellows (ESFY) disease that generally induces yellows, tree decline or die-back and vegetative disorders with typical symptoms such as an early bud break and leaf rolling on most wild and cultivated *Prunus* species (Poggi Pollini *et al.*, 2001).

ESFY is mainly known in Europe, but has also been reported in Turkey and Azerbajdzhan (Marcone *et al.*, 2010). Due to the high efficiency of its natural vector *Cacopsilla pruni* and the use of non symptomatic infected plant material, '*Ca. P. prunorum*' can spread rapidly in stone fruit cultivated areas. In apricot orchards the

number of infected trees can double in a few years, especially on susceptible varieties such as ‘Bergeron’ (Ramel and Gugerli, 2004, Poggi Pollini *et al.*, 2010).

To date, all approaches applied to plants already infected by phytoplasmas have not reduced the incidence of these epidemiological diseases (Osler *et al.*, 2012).

As a consequence, great relevance has been recently attributed to a well known phenomenon called recovery, the spontaneous remission of symptoms in diseased plants, intensively studied in apple and grapevine affected by phytoplasma and also known to occur in apricot infected by ESFY (Carraro *et al.*, 2004; Maixner, 2006a). Recovery can be natural or promoted by the application of abiotic stress, resistance inducers and several endophytic fungi and bacteria (Conti and Faoro, 2012); the involvement of a hypovirulent strain of ‘*Ca. P. prunorum*’ has also been suggested (Morvan *et al.*, 1986; Ermacora *et al.*, 2010).

In apricot orchards in Friuli-Venezia Giulia (Italy) under high ESFY infection pressure after several years of observation, three different groups of plants infected by ESFY were identified: symptomatic, symptomless and recovered (Osler *et al.*, 2012).

The aim of our work was to investigate the occurrence of recovery in 2 experimental orchards of apricot cv. Bergeron grafted on ‘Wavit’, located in the province of Trento (Italy), (Bleggio-Crosina and Bleggio- Farina). Both orchards were originally planted using ESFY-free material, but in the area ESFY has been spreading since 2000 leading to partial or total tree dieback and causing major economic losses to growers (Poggi Pollini *et al.*, 2010).

## Materials and Methods

Visual inspections for typical ESFY-symptoms (early bud breaks during dormancy, premature leaf-roll, fruit deformation and die-back) have been performed in the experimental orchards at least three times a year since 2006. All data were properly recorded appropriately.

Eighty-two apricot samples were analyzed using a Spot Real-Time Reverse Transcription-PCR Taqman assay, recently developed for ‘*Ca. P. prunorum*’ detection on stone fruit trees samples (Minguzzi *et al.*, 2010). Many samples showed typical ESFY-symptoms in late summer 2011; in contrast some did not show any visible symptoms. Five apricots infected by ESFY and 5 healthy apricots, grown in an insect-proof greenhouse, were also chosen as positive and negative controls, respectively. All molecular tests were performed during September-December 2011. All the samples, consisting of 1 g of phloem from woody shoots of apricot trees were placed in extraction bags (Bioreba, Reinach, Switzerland) and immediately tested or stored at -20C° until used. Analyses were performed using a rapid extraction method adapted from previously published works (Osman and Rowhani, 2006, Minguzzi *et al.*, 2010).

All the primers and probes employed in this study were designed using Primer Express software (Applied Biosystems, Branchburg, NJ, USA). Using sequence alignment of the 16S rRNA gene region of nine different phytoplasma strains (Baric and Dalla-Via, 2004), a specific ‘*Ca. P. prunorum*’ primer and MGB probe assay was designed.

A previously published assay (Osman *et al.*, 2007) was slightly modified in order to obtain a DiSTA 18s MGB probe and DiSTA 18s-F / DiSTA 18s-R primer pair.

Fluorogenic probes were 5' modified with FAM (6-carboxyfluorescein) (*ESFY-16S*) and VIC (*DiSTA 18s*). The multiplex RT-PCR reaction was performed in a total volume of 25  $\mu$ L containing 2  $\mu$ L of template, using an automated ABI PRISM 7000 Sequence Detection System (Applied Biosystems) in MicroAmp optical 96-well plates.

## Results

The results of field observations showed that three different groups of plants were present in the experimental fields. Many plants (57) expressed severe foliar symptoms, fruit deformation, progressive decline and sometimes total die-back in the last 2 years (2010-2011); according to previous results off-season growth was rarely observed (only 4 plants during winter 2011) (Poggi Pollini *et al.*, 2010). Some plants (13) showed ESFY-symptoms only in 2006-2007, but a spontaneous remission of symptoms has been occurring since 2009. The remaining 12 plants did never shown any visible ESFY-symptoms. Moreover in the last 2 groups no undersized fruits were observed and a good production was always achieved.

The distribution of the 3 groups in the 2 experimental orchards is summarized in Tab. 1.

As regards molecular tests, specific amplification was always obtained from the positive controls, from 57 symptomatic plants, from 13 recovered plants and surprisingly from 3 out of the 12 apparently healthy plants. No phytoplasma were found in the other 9 plants in the third group nor in samples from the healthy controls (Tab. 2).

Tab. 1 - different groups of plants collected in the experimental fields, during Sept-Dec 2011.

LOCATION AND NUMBER OF PLANTS	SYMPTOMATIC TREES	TREES WITH SYMPTOMS REMISSION	SYMPTOMLESS TREES
BLEGGIO – CROSINA (170)	26	6	6
BLEGGIO – FARINA (172)	31	7	6
TOTAL	57/342 – 16,7 %	13/342 – 3,8 %	12/342 – 3,5 %

Tab. 2 - results of phytoplasma detection by RT-PCR in different groups of trees.

LOCATION AND NUMBER OF PLANTS	SYMPTOMATIC TREES	TREES WITH SYMPTOMS REMISSION	SYMPTOMLESS TREES
BLEGGIO – CROSINA (170)	26/26*	6/6	1/6
BLEGGIO – FARINA (172)	31/31	7/7	2/6
TOTAL	57/57	13/13	3/12

\* number of samples positive by RT-PCR/total number of samples

## Discussion

The aim of our work was to select individual apricot with acquired tolerance in an area with a high ESFY-infection pressure.

Our research, based on a six-year plan of field inspections and on a rapid, sensitive and reliable diagnostic method (Spot Real-Time Reverse Transcription-PCR Taqman assay) clearly demonstrated for the first time the occurrence of recovery phenomena in 2 experimental apricot orchards in the province of Trento. Thirteen plants – 3.8% of the apricots in the experimental plots – stably recovered from the symptoms but not from the infection revealing acquired resistance. It is worth noting that usually the phytoplasma infection in the cv. Bergeron significantly reduces fruit size and causes fruit deformations leading to economic losses (Poggi Pollini *et al.*, 2010). In contrast these recovered plants did not show any fruit symptoms and produced a yield like the healthy plants. Interestingly three individual plants – less than 1% of the total-have remained symptom-less since 2006 even if infected; these plants can be considered tolerant to ESFY. All the studies conducted in Friuli-Venezia Giulia in apricot orchards provided clear evidence that ‘*Ca. P. prunorum*’ infections

persist in the aerial parts of recovered apricot trees (Osler *et al.*, 2012).

The potential epidemic threat posed by ‘*Ca. P. prunorum*’ in apricot orchards is confirmed by the dramatic increase in the number of infected trees, especially when a highly susceptible cultivar, like ‘Bergeron’, is grown under high European stone fruit yellows infection pressure (Poggi Pollini *et al.*, 2010). Like other phytoplasma, direct protection of trees from ‘*Ca. P. prunorum*’ cannot be achieved by chemical control measures. Moreover all the measures used to prevent ESFY spread such as sanitary selection, use of clean propagating material, removal of the infected plants and vector control with insecticide treatments have so far not reduced the progression of the disease in apricot orchards in Italy, (Poggi Pollini *et al.*, 2007; Osler *et al.*, 2012). Thus the possibility to develop new control strategies based on recovery phenomenon have recently become of practical interest (Conti and Faoro, 2012)

Recovery can be a temporary or a permanent phenomenon, thus it has been recently proposed that an originally infected plant can be considered as having recovered only after a minimum of 3 consecutive years without symptoms (Maixner, 2006b). As regards apricot and ESFY, in infective areas the infected but tolerant plants behave much better than healthy ones, when grafted on tolerant rootstocks. Moreover recovered plants are less likely to be re-infected than plants that have never been previously infected (Osler *et al.*, 2012). Despite in the fact that the physiological basis of recovery is still not completely understood and the interaction between hypo- and hyper-virulent strains of ‘*Ca. P. prunorum*’ in mixed infections is still under evaluation, the presence of recovered ESFY-infected plants has indicated the importance of this strategy also for the control of this infection (Ermacora *et al.*, 2010; Osler *et al.*, 2012).

In the next few years our research will focus on ascertaining the behavior of young plants derived from mother plants with acquired tolerance, grafted on appropriate rootstocks. A Real Time quantification method will be applied to confirm the correlation between low phytoplasma concentration and the absence of symptoms in the aerial parts of the trees (Ermacora *et al.*, 2010). The molecular identification of protectant strains which can be used for cross protection will also be performed.

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## **CLEAN PLANTS, THE NATIONAL CLEAN PLANT NETWORK AND HARMONIZING CERTIFICATION STANDARDS IN THE UNITED STATES**

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The National Clean Plant Network (NCPN) was established in the 2008 Farm Bill with three major objectives: a) develop and maintain G1 (Foundation) blocks that are to serve as sources of clean plant material for certification programs; b) carry out pathogen elimination in vegetatively propagated crops; and c) develop improved diagnostic tests for systemic pathogens. Limited funding was provided for four years to support and enhance existing facilities (clean plant centers), and development and validation of diagnostic assays. Currently the program supports a total of 19 programs at 16 centers working on five groups of perennial specialty crops: berries, citrus, grapes, hops, and tree fruits. A governing board was formed for each crop to develop lists of pathogens (viruses, phytoplasmas and systemic bacteria) that need to be tested, review proposals for funding, and coordinate activities between the centers. The program supports tree fruit service functions at Prosser, WA; Davis, CA; and Clemson, SC; and berry research and service functions in Corvallis, OR and Fayetteville, AR; and service functions in Raleigh, NC. In addition to efforts to develop these G1 blocks, the Farm Bill also provided funding to support certification programs, which in the U.S. are regulated at the state rather than the federal level. This funding is being used to harmonize certification programs across states for each of the crops. Ideally, the validation of diagnostic assays will be expanded to include international collaborators and many aspects of the certification standards being developed will be harmonized with programs in other countries.

## ESTABLISHMENT OF VIRUS-FREE PLANTING MATERIAL FOR POME FRUITS IN LATVIA

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Over the past twenty years, many commercial apple orchards have been established by the use of introduced cultivars and clonal rootstocks. Due to the specific requirements of adaptation to local climatic conditions, mainly domestic cultivars and cultivars originating in Russia, Belarus and Kazakhstan are grown. In Latvia a certification programme for virus-free planting material has not been established yet and planting materials of pome fruits correspond to *Conformitas Agraria Communitatis* standard. In order to establish a nuclear stock collection in the country for several widely grown pome fruit cultivars candidate graft materials from four apple cultivars 'Auksis', 'Antei', 'Antonovka', 'Sinap Orlovskii' and six pear genotypes 'Belorusskaya Pozdnaya', 'Conference', 'Concorde', 'Condo', 'Vasarine Sviestine', P-67-21 were budded on seedling rootstocks grown in a sterilized substrate and insect proof greenhouse. the following year before thermotherapy common viruses were detected by multiplex RT-PCR. All apple cultivars were infected with *Apple chlorotic leaf spot virus* (ACLSV). Cultivars 'Auksis' and 'Sinap Orlovskii' were also infected with *Apple stem pitting virus* (ASPV) and *Apple stem grooving virus* (ASGV), but cultivar 'Antei' was infected with ASPV. All pear cultivars were infected with ApMV and cultivar 'Conference' was also infected with ASPV and ASGV. In order to eliminate the viruses the plants were kept in a growth chamber for 40 days at temperature of  $38 \pm 0.5^{\circ}\text{C}$ . The shoot tips (5 mm) were excised from heat treated plants and grafted onto a seedling rootstocks. Apple cultivars 'Auksis' and 'Antonovka' were negative for all tested viruses during two vegetation periods. Cultivars 'Antei' and 'Sinap Orlovskii' remained infected with ASGV, which was detectable only in the second year after thermotherapy. Pear cultivars 'Belorusskaya Pozdnaya' and 'P 67-21' remained infected with ApMV. Work is in progress to test candidate mother plants by woody indicators for viruses and other graft-transmittable organisms included in EPPO guidelines for pome fruits.

## **DEVELOPMENT OF MOLECULAR DIAGNOSTIC TOOLS TO DETECT ENDEMIC AND EXOTIC PATHOGENS OF *PRUNUS* SPECIES FOR AUSTRALIA**

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The biosecurity of the Australian almond, summer fruit and cherry industries is maintained at the border by the Australian Quarantine Inspection Service (AQIS) in Post Entry Quarantine (PEQ) facilities and internally through schemes that supply high-health planting material throughout Australia. Currently the AQIS recognizes two bacteria, six phytoplasmas or phytoplasma-like diseases, 13 viruses, one viroid and 70 fungi, to be of quarantine significance for *Prunus* species. AQIS also recognizes that there are many diseases of *Prunus* of unknown aetiology. A recent review of the bacteria, phytoplasmas, viruses, viroids and fungi that are currently known to infect *Prunus* species identified the pathogens that are significant to Australian PEQ: five bacteria are significant at the quarantine level and another four are significant at the plant health certification level within Australia. 11 phytoplasmas or phytoplasma groups are known to infect *Prunus* species and no phytoplasmas have been reported to infect *Prunus* species within Australia. 34/47 viruses that are known to infect *Prunus* species are significant at the PEQ level. Only one of three viroids that infect *Prunus* species are reported as significant at the PEQ level. Based on these findings a new research project aimed at improving Australia's stone fruit biosecurity is underway to: Update the PEQ list for *Prunus* species with recently reported pathogens and updated information of known pathogens. Adopt, develop and validate molecular diagnostic tools under Australian conditions for the important quarantine pathogens of *Prunus* species. Develop a PEQ diagnostic manual for a number of *Prunus* species. The development of these molecular protocols will support industry biosecurity plans that have been developed for Australian almonds, summer fruit and cherry industries.

## QUALITY SYSTEMS FOR PRODUCTION OF NURSERY STOCK IN APPLE

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Growers of nursery stock aim to produce a high quality product. Plants are selected and propagated, stocks are indexed and possibly certified through quality-plus systems. Detection tests for plant pathogens e.g. viruses are carried out in order to remove infected plant material. Within quality plus systems several techniques are combined to detect a range of virus(like) diseases, but increasingly the focus has shifted to molecular testing. Although molecular detection tests are rapid, they are generally aimed at a specific target and might therefore miss (new) variants of a virus. In recent years laboratories like inspection services have started to obtain accreditation for the detection tests. Validation is the key to describing the reliability of a detection test. As an example we will focus on screening for the presence of *Apple stem pitting virus* (ASPV), *Apple stem grooving virus* (ASGV), *Apple chlorotic leaf spot virus* (ACSLV) and *Apple mosaic virus* (ApMV). Comparison of RT-PCR's – published and modified primer sets – testing by grafting on indicators, and adjustments needed for routine testing of samples will be discussed in relation to quality plus systems and the availability of virus collections.

## **ESTABLISHMENT, ACTIVITIES AND RESULTS OF PLANT BIOTECHNOLOGY LABORATORY IN KABUL, AFGHANISTAN**

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The horticultural industry is a vital component of the agricultural sector of Afghanistan and a primary engine of Afghanistan's recovering economy is its agriculture, which engages approximately 80% of the working population. This sector was thriving in the 1970s, but is today incapable of competing in the international market. To recover and develop the horticulture sector of the country, EC supports PHDP (Perennial Horticulture Development Project), to provide true to type/ecotype and healthy planting materials, and the Plant Biotechnology Laboratory to ensure the health status of local germplasm. This laboratory started screening the health status of the Afghan Germplasm National Collection in order to ensure the multiplication of not only the best selected varieties or ecotype, but also to avoid the production and distribution of virus-infected fruit trees. Symptom inspection and sample collection for viral diseases was carried out in all the National Collection fields, including peach, plum, apricot, almond, apple, grape and citrus plants, located in various areas of the country (Kabul, Kunduz, Mazar, Herat, Kandahar and Jalalabad). During the routine activities of the laboratory, citrus plants from the National Collection experimental farm in Jalalabad (Nangarhar province) showing vein flecking, yellowing and plant decline symptoms were found to be infected by *Citrus tristeza virus* (CTV). Identified isolates were characterized molecularly by amplifying a fragment corresponding to the major coat protein gene from all positive samples. Moreover *Prunus necrotic ringspot virus* (PNRSV) was detected in samples collected from three almond accessions from the PHDP research station of Mazar Sharif (northern Afghanistan). To our knowledge, our results identified for the first time CTV and PNRSV in Afghanistan. The presence of these viruses in different accessions of the National Collection is of concern for Afghan horticulture. Implementation of the certification schemes is therefore necessary to quarantine the production and the employment of virus-free propagating material.

## CAV'S LABORATORY ACTIVITY WITHIN THE ITALIAN CERTIFICATION SCHEME OF PLANT PROPAGATION MATERIAL

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CAV (Centro Attività Vivaistiche) is an association of nurserymen founded in 1982. Its main goal is to improve the quality of the nursery production by genetic and phytosanitary certified propagating materials. With more than 800 primary sources grown in its screen-houses, CAV represents the main Nuclear and Propagation Centre in Italy, officially recognized by the Italian Ministry of Agriculture (Mipaaf) to supply pome fruits, stone fruits, strawberry and olive prebasic and basic propagating material, to Italian nurseries as well as to other international customers. To guarantee its procedures and products, CAV obtained the ISO 22005:2008 certification for row traceability. In order to fulfill all the technical requirements listed in the Italian certification scheme, CAV manages infrastructures (glass houses) and a modern laboratory, accredited by Mipaaf, equipped for the detection of several pests (viruses, bacteria, fungi, phytoplasmas, viroids, mites and insects) and for the assessment of cultivar trueness-to-type (fingerprinting) for candidate primary source, prebasic, basic and certified material. The techniques currently used include biological (indexing), serological (ELISA, IFAS), molecular (PCR, Real-Time PCR), microbiological tests (isolation and identification) and microscopy observations (optical/fluorescence). CAV's laboratory is also equipped to obtain virus free clones of fruit varieties by several methods i.e. heat therapy and tissue culture.

The 2011 activities include:

2296 biological assays

2802 serological tests for primary source, pre basic and basic material

11918 serological tests for certified material

1233 molecular tests for primary source, pre basic and basic material

149 selected/sanitized primary sources submitted for registration in the National certification scheme

The increasing needs arising from the internationalization of its activities and the improvement of the quality management system led CAV in 2009 to request accreditation in agreement with ISO/IEC Standard 17025 *General requirements for the competence of testing and calibration* (credit ILAC/ ACCREDIA. No. 0896). In the same period, CAV obtained the official recognition by MAF BIOSECURITY NEW ZEALAND for testing Malus and Pyrus 'high healthy material' to be exported to New Zealand.

## **CIVI-ITALIA, THE ITALIAN NURSERY ASSOCIATION AND ITS ROLE WITHIN THE FRAMEWORK OF THE ITALIAN CERTIFICATION SCHEME**

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CIVI-Italia, the inter-professional centre for nursery activities, is the national consortium among nursery groups and growers' union associations. It was officially recognized by the Italian Ministry of Agriculture (Mipaaf) with a ministry decree issued on December 2nd, 1993, as the exclusive national association to represent the nursery sector for promoting and carrying out the plant propagating certification programme in Italy. CIVI-Italia plays an important role within the framework of the Italian voluntary certification scheme through its main statutory objectives of enhancing nursery production in order to qualify vegetable, fruit, olive and citrus yields. For the tasks assigned to it, CIVI-Italia facilitates connections among the various organizations that make up the National Certification Scheme which is composed by

9 Centers of conservation for premultiplication, located in 7 different regions;

10 centers of premultiplication, located in 8 different regions;

26 centers of multiplication (mother blocks), extended over 150 ha, located in 10 different regions;

Over 100 nurseries which manage nursery plots extended over 2.500 Ha

1511 Primary sources of the varieties most requested by the market.

Upon completion of administrative and field checks, the relevant Regional Phytosanitary Service notifies certification admission to the nurseryman and to the body charged of printing the label certificates, together with the authorisation to print and affix the label/certificate. Labels/certificates are made and distributed by CIVI-Italia, who send the final schedules showing the numbers assigned to the labels/certificates to the local Regional Plant Health Services and to the Ministry for Agriculture and Forestry Policies every year. In the last season 2011/12, the volume of certified plants produced in Italy by 102 nurseries was made up of: 6.83 pome fruit; 1.24 stone fruit; 22.3 fruit rootstocks; 190 strawberry (millions of plants).

*The certification program in Italy, following the new course set up in 2003, constitutes a positive experience implemented under the coordination and control of the Ministry and Regional Phytosanitary Services, in strong cooperation with the private nursery sector.*

## **THE VOLUNTARY CERTIFICATION SCHEME FOR FRUIT PLANT PROPAGATING MATERIAL IN ITALY**

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In Italy, the qualification of plant propagating material, with the establishment of certification programmes has been carried out at regional level in various Italian regions for different species since 1981. The rules adopted were quite different although referring to very similar protocols and in agreement with the international regulations. The Italian National Service for the Voluntary Certification was founded in 1989 by a ministerial decree. Over the years, some regions participated in the national certification programs for pome and stone fruit, olive, strawberry and citrus. Due to the occurrence of new pests and diagnostic techniques, and on the basis of these experiences, the necessity to modify the rules was clear, also in the light of the European Directive 92/34/EEC, which was established in Italy in April 1997. Furthermore, the Regional Governments in Italy were assigned the tasks previously fulfilled by the Central Administration. Between 2003 to 2006, new decrees reorganized the National Certification Service and in 2006 updated technical protocols for each species were issued. In accordance with these rules, the National Certification Service has been established at the Ministry for agriculture food and forestry policies (Mipaaf) and is in charge of national coordination.

It is composed of: The Technical Committee, Operating Secretariat and Regional Phytopathological Services.

The Regional Phytopathological Services are in charge of the certification (sanitary and trueness to type controls) in their territory. There are 1.511 officially recognized primary sources in Italian certification scheme, in representing the classic and most recent varieties licensed by the national and international breeding programs. In the last season 2011/12, the volume of certified plants produced in Italy by 102 nurseries was made up of: 6,83 pome fruit; 1,24 stone fruit; 22,3 fruit rootstocks; 190 strawberry (million of plants).

## GLOBAL GENE EXPRESSION ANALYSIS OF PHYTOPLASMA IN THE HOST SWITCHING BETWEEN PLANT AND INSECT

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### Abstract

Phytoplasmas are intracellular parasites of both plants and insects, and are spread among plants by insects. Here, we show the global gene expression analysis of ‘*Candidatus Phytoplasma asteris*’ OY-M strain. As a result, the expressions of genes for transporters, secreted proteins, and metabolic enzymes were suggested to be altered in response to the plant and insect host. These results suggest that the phytoplasma may use these genes in a host-specific manner.

### Introduction

Phytoplasmas are unique biologically in that they can parasitise a diverse range of hosts, including plants and insects (Firrao *et al.*, 2007; Hogenhout *et al.*, 2008). Phytoplasmas can reside endocellularly within the plant phloem and feeding insects (leafhoppers), and are spread among plants by insects (Christensen *et al.*, 2005). It is of interest how phytoplasmas can adapt to two diverse intracellular environments (i.e., plant and insect cells). We have previously demonstrated that a complex between Amp, a surface membrane protein of phytoplasma, and insect microfilament may play a major role in the insect transmissibility of phytoplasma (Suzuki *et al.*, 2006). However, the mechanisms enabling the switch between plant and insect hosts are poorly understood. To investigate the gene expression levels in the phytoplasma, we designed and constructed a microarray based on the genomic sequence (Oshima *et al.*, 2004) of *Candidatus Phytoplasma asteris*, OY strain, OY-M line, and performed the global gene expression analysis.

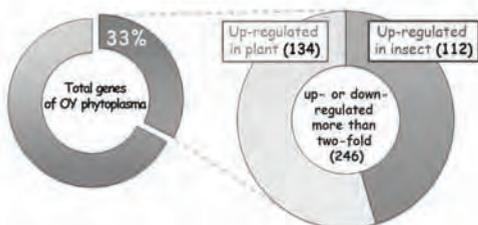


Fig. 1 - Global gene expression profiling of the phytoplasma in plant and insect hosts. 246 genes (ca. 33% of the genes in the genome) were differentially expressed between the two conditions. 134 genes were upregulated in the plant host, whereas 112 genes were upregulated in the insect host.

## Materials and Methods

The mild symptom line of 'Candidatus Phytoplasma asteris' OY strain (OY-M) was maintained in garland chrysanthemum (*Chrysanthemum coronarium*), using the leafhopper vector *Macrostelus striifrons* (Oshima *et al.*, 2001). Total RNA was extracted from OY-infected plants and OY-infected insects (Himeno *et al.*, 2011), labeled with Cy3 and Cy5, respectively, and used for microarray analysis. Expression levels obtained by microarray analysis were checked by qRT-PCR, in which the expression level of *tufB* gene was used as an internal standard.

## Results and Discussion

To investigate the molecular mechanism underlying the 'host switching' between plant and insect, we performed the global gene expression analysis of *Candidatus Phytoplasma asteris* OY-M strain. As a result, ca. 33% of the genes in the total genome were differentially expressed between the two conditions (Fig. 1). The phytoplasma alters many gene expressions in response to the plant and insect host, such as genes for transporters, secreted proteins, and metabolic enzymes. These results suggest that the phytoplasma may use transporters, secreted proteins, and metabolic enzymes in a host-specific manner.

As phytoplasmas reside within the host cell, the proteins secreted from phytoplasmas are thought to play crucial roles in the interplay between phytoplasmas and host cells (Hoshi *et al.*, 2009; MacLean *et al.*, 2011; Sugio *et al.*, 2011). For example, one of the secreted proteins, TENGU, induces witches' broom and dwarfism. The *tengu* gene is up-regulated in the plant host, suggesting that this protein may function mainly when the phytoplasma grows in the plant host (Hoshi *et al.*, 2009). Our microarray analysis revealed that the expression of the gene encoding the secreted protein PAM486 was highly upregulated in the plant host, which is also observed by immunohistochemical analysis (Oshima *et al.*, 2011), suggesting that this protein functions mainly when the phytoplasma grows in the plant host.

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## DETECTION AND IDENTIFICATION OF A PHYTOPLASMA AFFECTING BLACK CURRANT AND RED CURRANT SHRUBS IN NORTH AMERICA

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### Abstract

During October-November/2011, blackcurrant (*Ribes nigrum* L.) and redcurrant (*Ribes rubrum* L.) shrubs from Ontario, Canada that exhibited symptoms of leaf redness and downward curling were tested to verify phytoplasma presence. Total DNA was extracted and used in a nested PCR assay with universal primers that target the phytoplasma 16S rRNA gene in nested reaction. Amplicons were obtained for all the symptomatic plants collected and phytoplasma sequence from both blackcurrant and redcurrant plants showed a 99% sequence identity to those of members of group 16SrX, subgroup 16SrX-A. Virtual RFLP of the R16F2n/R2 amplicons yielded patterns identical to those of 'Ca. P. mali' (AJ542541). Phylogenetic analysis based on the 16S rDNA sequences confirmed RFLP results. Phytoplasmas of group 16SrX have been previously reported in Ontario, affecting *Prunus* and *Pyrus* species. However, our results represent the first record of a 16SrX-A infecting blackcurrant and redcurrant in Canada and North America, and provide a valuable tool for further epidemiological studies of a 16SrX phytoplasma in these two plant species.

### Introduction

*Ribes nigrum* L. and *Ribes rubrum* L. are known as perennial edible berries, with a high content in vitamin C (ascorbic acid) and antioxidants, and native to central, western, and northern Europe and northern Asia. About 825,000 t (908,000 tons) of *Ribes* fruit are produced worldwide, almost entirely in Europe for natural consumption and juice production (Dale, 2000). *Ribes* species have been widely used for traditional herbal medicine as diuretics, diaphoretics and antipyretics, and to treat asthma, allergies, arthritis, adrenal atrophy, migraine, skin disorders and sore throats. Moreover, recent new findings highlight the use of *Ribes* species as good dietary sources with potential antidiabetic and antihypertension properties (Da Silva Pinto *et al.*, 2009).

Particularly, in North America, various *Ribes* species are native from northern Canada (Hudson Bay) and Alaska, south to California, New Mexico, Arkansas and North Carolina (Dale, 2000). Fruit from black, red and white currants, and gooseberries (*Ribes* L.) were grown commercially in North America at the beginning of the 20th century. Nowadays, commercial acreage of *Ribes* in North America is located where the growing day degrees are above 5°C (41°F), and the annual chilling hours are at least 1200 (Dale, 2000). Blackcurrants are more popular in Canada and the northern states and redcurrants and gooseberries are of more interest further south. Present estimates of production would suggest that several hundred acres might be grown throughout North America, mostly in Ontario, British Columbia, Oregon and Washington. Now a wide range of imported *Ribes* products is available particularly in Canada, and the pick-your-own (PYO) market is increasing.

A number of pests and diseases have been listed affecting black currant and red currant plants in North America, including the black currant reversion disease (BRD), which is considered the most destructive disease of black currants worldwide (Jones,

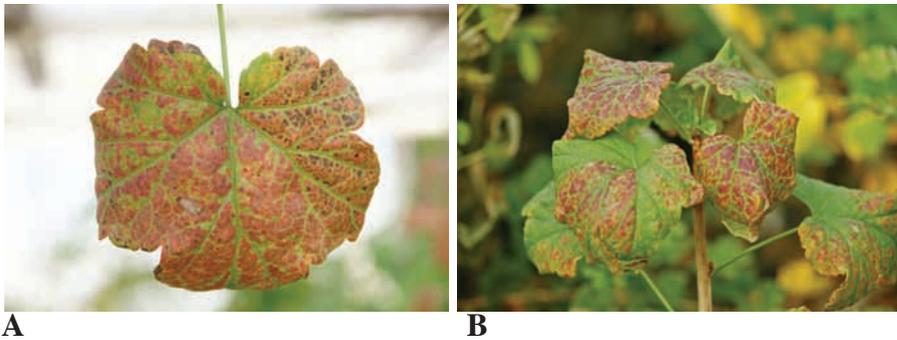


Fig. 1-Symptoms of leaf redness (Fig A, B) and down curling (Fig B) exhibited by black currant plants in Ontario, Canada.

2002). Although BRD has been mostly associated with a nepovirus, phytoplasmas have been also detected in BRD-affected plants particularly in Europe, including Czech Republic, Finland, and Russia (Lemetty *et al.*, 2007; Spak *et al.*, 2004; Navratil *et al.*, 2007).

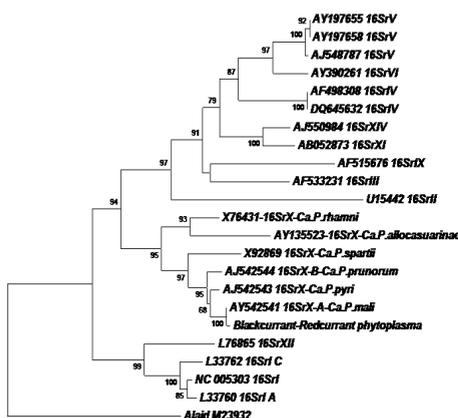


Fig. 2 - Phylogenetic tree showing relationships between the phytoplasma identified in black currant and red currant plants in Ontario, Canada. ‘Ca. P.’ stands for ‘*Candidatus* Phytoplasma sp.’; Alaid: *Acholeplasma laidlawii*; 16SrI: ‘*Ca. P. asteris*’; 16SrII: ‘*Ca. P. aurantifolia*’; 16SrIII: X-disease; 16SrIV: lethal yellowing; 16SrV: ‘*Ca. P. ulmi*’; 16SrVI: ‘*Ca. P. trifolii*’; 16SrIX: pigeon pea witches’ broom; 16SrXI: ‘*Ca. P. oryzae*’; 16SrXII: ‘*Ca. P. australiense*’; 16SrXIV: ‘*Ca. P. cynodontis*’

Phytoplasmas are the primary limiting factors for more than 600 herbaceous and woody plants species worldwide including many important food vegetable , and fruit crops; ornamental plants; and timber and shade trees (Bertaccini and Duduk, 2009). The present study presents the first results on detection and identification of a phytoplasma affecting black currant and red currant in Ontario, Canada.

### Materials and Methods

During October-November/2010, in the Ontario province of Canada, leaf midribs from plants of blackcurrant and redcurrant that mostly exhibited leaf redness and downward curling (Fig. 1) were collected from black and red currant fields in Ontario. Five plants were sampled in total, including a non-symptomatic plant.

Total DNA was extracted from 100 mg midribs (FastDNA Spin kit, MP Biomedicals, USA) and used as a template in a nested PCR with primers P1/P7 (Deng & Hiruki, 1991, Smart *et al.* 1996) followed by either R16F2n/R2 (Gundersen & Lee, 1996; Smart *et al.*, 1996) or fU5/rU3 (Lorenz *et al.*, 1995). PCR was performed with illustra Pure Taq Ready-to-go-PCR-beads (GE Healthcare, UK), and a DNA Engine Peltier thermal cycler Chromo 4 (Biorad). The PCR products were purified (Cycle Pure Kit, Omega bio-Tek, USA) and directly sequenced (Sequencing Service, University of Health Network, Toronto, Canada). The 16S rDNA sequence was subjected to phylogeny (MEGA version 3.1), in comparison to those of reference phytoplasmas in GenBank (<http://www.ncbi.nlm.nih.gov>), as well as *in silico* RFLP (pDRAW32, (<http://www.aacalone.com>) with restriction endonucleases *AluI*, *BstUI*, *HaeIII*, *HhaI*, *HpaII*, *MseI*, *RsaI*, *DdeI*, *Tsp509I*, *TaqI* and *SspI*.

### Results and Discussion

Phytoplasma DNA was detected in 2 out of the 3 black currant plants and in one out of one of the redcurrant plants collected. No DNA was detected for the non-symptomatic plant. Symptoms observed in both black and red currant plants were unique and differed from those previously reported for BRD (Lemetty *et al.*, 2007; Spak *et al.*, 2004; Navratil *et al.*, 2007), and include yellowing and proliferation, leaf-like structures or flower abnormalities narrower leaves with reduced number of main veins, larger but fewer marginal serrations, and a basal sinus that is less lobed.

The phytoplasma 16S rDNA sequence obtained from both the black and red currant plants shared a 100% sequence identity to each other and showed a 99% sequence identity to those of phytoplasma members of group 16SrX, 'apple proliferation'. The consensus sequence was deposited in GenBank under the accession number (JX847650). These results were fully supported by the phylogenetic analysis since the phytoplasma identified in both black and red currant plants clustered within the 16SrX phylogenetic branch, more closely related to the '*Candidatus* Phytoplasma mali' strain (AJ542541) (Fig 2).

The *AluI*, *BstUI*, *HaeIII*, *HhaI*, *HpaII*, *MseI*, *RsaI*, *DdeI*, *Tsp509I*, *TaqI* and *SspI* RFLP patterns of the 16S rDNA sequence of the phytoplasma detected in both black

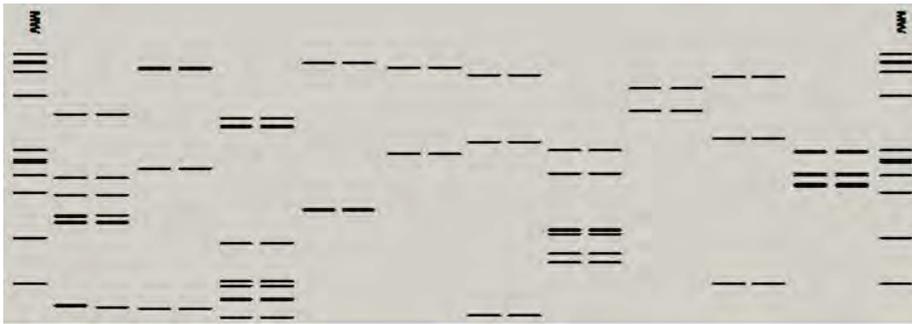


Fig. 3 - *AluI*, *BstUI*, *HaeIII*, *HhaI*, *HpaII*, *MseI*, *RsaI*, *DdeI*, *Tsp509I*, *TaqI* and *SspI* *in silico* RFLP patterns from R16F2n/R2 PCR amplicons corresponding to the consensus sequence of the phytoplasmas identified in both black and red currant plants, and ‘*Ca. P. mali*’ (AJ542541), subgroup 16SrX-A. Lanes 1, 24: phiX174 DNA-*HaeIII* Digest molecular marker; Lanes 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22: RFLP patterns for *Ribes* phytoplasmas; Lanes 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23: RFLP patterns for phytoplasma strain AJ542541.

and red currant plants were identical to those exhibited by the phytoplasma reference strain (AJ542541) that belongs to subgroup 16SrX-A (Fig. 3), which confirmed the phylogeny results and the fact that the phytoplasma detected in both *Ribes* species was also a 16SrX-A member.

Two main groups of phytoplasmas have been identified in *Ribes* species affected by BRD in Europe: group 16SrI (‘*Ca. P. asteris*’) in Czech Republic, Finland, and Russia (Lemetty *et al.*, 2007; Spak *et al.*, 2004; Pribylova *et al.*, 2007) in black currant; and group 16SrX in red and white currant plants in Czech Republic (Navratil *et al.*, 2007), the latter in association with phytoplasmas belonging to group 16SrI.

The 16SrI is the group with the highest plant host range worldwide and was associated with major economic losses of vegetable crops (including lettuce, carrot, and celery) and ornamental plants (including gladiolus, hydrangea, China aster, and purple coneflower) in North America and Europe (Bertaccini and Duduk, 2009). This phytoplasma group has also been reported in Canada in a number of plant species (Olivier *et al.*, 2009), including 16SrI phytoplasma potential vectors like *Graminella nigrifrons* (Arocha *et al.*, 2011).

The phytoplasmas of group 16SrX are of high impact in Europe; just one 2001 phytoplasma outbreak in apple trees caused a loss of about 25 million euros in Germany and about 100 million euros in Italy (Strauss, 2009). The subgroup 16SrX-A has been mainly identified in apple trees affected by apple proliferation disease, which highlights a highly specific host-pathogen relationship (IRPCM, 2004).

In Canada, the subgroup 16SrX-C has been reported in pear (Hunter *et al.*, 2010) and peach (Arocha *et al.*, 2011). The subgroup 16SrX-A was previously reported in *G. nigrifrons* (Arocha *et al.*, 2011) but not in plant hosts. No phytoplasmas have been reported in *Ribes* species in North America, therefore the fact that group 16SrX has just been confirmed is of significant impact for the re-emerging *Ribes* sp. industry in Canada since new disease management strategies need to be implemented to halt disease spread, and provide a more effective control of phytoplasma diseases.

Results refer to the first report of phytoplasmas in *Ribes* sp. in North America, and the identification of the subgroup 16SrX-A affecting blackcurrant and redcurrant plants. Studies are required to identify the potential vectors for the 16SrX-A phytoplasma, and to determine a vector role and ecological factors influencing the transmission of phytoplasma diseases in *Ribes* sp, which will contribute to improve phytoplasma disease control in Canada.

#### Acknowledgements

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## MOLECULAR POLYMORPHISM IN PHYTOPLASMAS INFECTING PEACH TREES IN SERBIA

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During a survey carried out in 2008 to verify phytoplasma presence in fruit trees in Serbia, samples were collected from two individual peach plants (30-Niš and 103-Radmilovac) showing yellows disease. Phytoplasma detection was carried out using PCR assays with primer pair P1/P7 in direct reaction, followed by nested PCR with F1/B6, R16F2n/R2, and R16(I)F1/R1. RFLP analyses carried out on R16F2n/R2 amplicons with *Tru*II, *Taq*I, *Tsp509I*, *Alu*I, and *Bfa*I restriction enzymes showed that in peach 30, a mixed phytoplasma infection was present. Identification of 16SrII and 16SrXII phytoplasmas was confirmed by RFLP analyses carried out on amplicons obtained with primers R16(I)F1/R1 with *Tru*II and *Tsp509I* restriction enzymes. RFLP analyses on F1/B6 and R16F2n/R2 amplicons with *Tru*II showed that 16SrII group phytoplasmas were present also in peach 103. F1/B6 and R16F2n/R2 amplified products (about 1,700 bp and 1,200 bp respectively) of both peach samples were purified using Qiagen PCR Purification Kit, cloned in DH5 and a number of clones were screened by PCR with M13 primers followed in nested PCR by F1/B6 and R16F2n/R2 primers (according with amplicon). RFLP analyses with *Tru*II on R16F2n/R2 amplicons of seven M13 clones obtained from peach 30 showed the presence of 6 profiles referable to 16SrXII and two referable to 16SrII phytoplasmas. *Alu*I restriction enzyme did not show polymorphisms but distinguished between groups 16SrII and 16SrXII while *Tsp509I* showed different profiles between the two 16SrII group phytoplasmas. Similar results were obtained with *Tru*II and *Alu*I restriction enzymes on 3 clones amplified with R16(I)F1/R1. Peach 103 clones were all identical after RFLP analyses. This is the first report of 16SrII phytoplasmas in peach and the 16Sr DNA variability detected in both 16SrXII and 16SrII phytoplasmas is an indication of phytoplasma population presence in the infected plant.

## **SEROLOGICAL PROOFS FOR RELATEDNESS OF THE MYCOPLASMA-LIKE ORGANISMS (PHYTOPLASMAS) FROM APPLE PROLIFERATION GROUP**

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Two polyclonal antibodies were produced in rabbits for apricot chlorotic leaf roll (ACLR) MLO purified from apricot, and for European Aster yellows (EAY) cultivated in artificial media. Both antisera were used as the primary antibody to detect apple proliferation (AP15), pear decline (PD) and ACLR, in a comparative study with EAY MLO purified from periwinkle. AP15 (from Udine, Italy) was kindly provided by E. Seemüller, Germany, and in our laboratory, it was identical by symptoms (virescence and proliferation) in periwinkle to EAY isolated in Romania and with American severe western yellows. An indirect dot blot ELISA method was performed on nitrocellulose membrane stripes using secondary antibodies Goat anti-rabbit IgG conjugated with Alkaline Phosphatase (Sigma product No. A3937) and Goat anti-rabbit IgG conjugated with colloidal gold (Sigma product No. G3779). Positive reactions were identified for alkaline phosphatase using SIGMAFAST-BCIP/NTB (Sigma product No. B0274 substrate). When EAY and AP15 MLOs purified from periwinkle were tested with antiserum for ACLR, these gave a positive reaction. Pear decline MLO extracted from pepper infected by *Cacopsylla pyri* and from pear trees was also detected with anti ACLR MLO serum. MLO purified from *C. pyri* insects used in experimental transmissions was also detected. These results support the hypothesis that EAY, AP15, PD and ACLR MLOs are identical serologic. The EAY antiserum recognized AP15 and EAY MLOs (phytoplasmas) purified from periwinkle when both molecular markers (AP and Colloidal gold) were used. The MLOs from AP15 and AP15 group (PD, ACLR) were also identical with EAY which infect more than 500 species of cultivated and spontaneous plants. A recent investigation in Germany, based on PCR analyses of 16S ribosomal DNA revealed that AP, PD and ACLR are identical pathogens.

## MULTILOCUS GENE ANALYSES OF ‘*CANDIDATUS PHYTOPLASMA MALI*’ CONFIRMS THE GENETIC DIVERSITY OF PHYTOPLASMA POPULATION IN THE CZECH REPUBLIC

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Apple trees are affected by the apple proliferation (AP) disease in several European countries. The AP is usually associated with the presence of ‘*Candidatus Phytoplasma mali*’ (‘*Ca. P. mali*’), a quarantine pathogen in EU. Samples from several apple trees showing proliferation disease symptoms were collected during 2010 at 28 different locations all over the Czech Republic. Phytoplasma strains were detected through polymerase chain reaction and restriction fragment length polymorphism analyses of 16S rDNA-spacer region-23rDNA, ribosomal protein gene sequences *rpl22* and *rps3* and non ribosomal DNA fragment (nitroreductase-like gene). All 74 apple trees examined were positive for phytoplasmas, predominantly for ‘*Ca. P. mali*’ (60 trees). ‘*Ca. P. asteris*’ alone and in also mixed infection with ‘*Ca. P. mali*’ was detected sporadically. In the 16S plus spacer region of ‘*Ca. P. mali*’ two profiles P-I (39 trees) and P-II (5 trees) singly or together (8 trees) were identified. The presence of two genetic lineages designated in the literature as pattern ‘1’ (7 trees) and ‘2’ (31 trees) individually and also mixed (7 apple trees) was detected while the only pattern named ‘a’ was identified in 45 apple trees. ‘*Ca. P. mali*’ strains belonging to ribosomal protein rpX-A subgroup were identified in the majority of apple samples (51 out of 55 plants positive in direct PCR amplification with rpAP15f/rpAP15r primers), while phytoplasmas belonging to rpX-B subgroup were detected sporadically (four trees). Nearly equal distribution of apple proliferation subtypes AP-15 and AT-2 (in 21 and 22 trees, respectively) was determined, while apple proliferation subtype AT-1 was detected in 7 trees. Nucleotide sequence analyses of the 16S-23S ribosomal operon, ribosomal proteins L22, S3 and nitroreductase-like protein gene of five selected apple proliferation phytoplasma strains confirmed the PCR/RFLP analyses results. This is the first study of molecular diversity among ‘*Ca. P. mali*’ strains in the Czech Republic.

## **EARLY AND RELIABLE DETECTION OF EUROPEAN STONE FRUIT YELLOWS PHYTOPLASMA IN PEACH TREES**

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The presence of European stone fruit yellows (ESFY), syn. '*Candidatus* Phytoplasma pruni' detection in peach trees grown in South Moravia, Czech Republic was studied during a five year period. ESFY symptoms were evaluated visually and biological indexing was carried out on the peach indicator GF-305. Three different procedures of molecular detection of ESFY were used. The first was PCR with primers ECA1/ECA2 according to Jarausch *et al.* (1998), the second one with primers fAT/rPRUS according to Smart *et al.* (1996), and third procedure was nested PCR with primers R16R0/R16F1 in the first step and primers R16F2/R16R2 in the second step according to Lee *et al.* (1995). Detection of ESFY in peach trees by biological indexing was found to be less suitable, the presence of the pathogen was proved in less than 60% of symptomatic trees. Results of the all three applied PCR procedures were the same, but reactions of nested PCR were stronger. The presence of ESFY was detected in ca 75% of symptomatic trees. Results of PCR were negative in symptomless peach trees. The presence of ESFY symptoms is still the most reliable criterion for the detection of ESFY disease in peach trees grown in the Czech Republic. Further improvement of PCR procedures is necessary for early and reliable detection of ESFY in peach trees.

## DETECTABILITY OF PHYTOPLASMAS IN NATURALLY INFECTED *PICEA* AND *PINUS* SPP. TREES BY PCR

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Coniferous plants in Europe are natural hosts of three phytoplasmas - '*Candidatus* Phytoplasma asteris', phytoplasma belonging to X-disease phytoplasma group and '*Ca. P. pini*'. Very recently '*Ca. P. pini*' was identified in trees and shrubs of eight species. Unfortunately, the previous studies yielded unsatisfactory results concerning phytoplasma detection in coniferous plants. This work was undertaken to study year round detectability of phytoplasmas in the needles of naturally infected coniferous plant species and to examine detectability of '*Ca. P. pini*' in plant material stored at different conditions (fresh needles, needles dried at room temperature and stored at 20°C or -18°C, lyophilized material stored at 4°C and frozen needles stored at -70°C). Experiments with phytoplasma infected pine and spruce trees indicated that phytoplasma detection measured by PCR assay was different depending on the host plant as well as time of testing. In *Picea tabuliformis* tree, '*Ca. P. pini*' was detected in samples of needles collected every month. In contrast, in genotypes such as *Picea pungens* and *P. abies*, phytoplasma detectability was lower. In two *P. abies* trees infected with phytoplasma X-disease, the pathogen was detected in 70% of the tested samples mainly in nested PCR using P1/P7 followed by R16F2n/R16R2 primer pairs. In two *P. pungens* trees affected with '*Ca. P. pini*' phytoplasma was detected only in spring and summer months. In affected *Pinus banksiana* and *P. sylvestris* plants '*Ca. P. pini*' was detected in direct PCR in fresh plant material as well in needles stored for 12 months at -70°C or stored at 4°C after lyophilization. Detectability of phytoplasma in needles dried at room temperature and stored at -18°C or at 20°C for 12 months was relatively lower; in these samples phytoplasma was only detected only by nested PCR.

## FIRST OCCURRENCE OF PEAR DECLINE DISEASE IN PORTUGAL

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All over the world there are over a thousand phytoplasma-associated diseases known, causing high economic losses. They affect all kinds of plant species, such as vegetables, fruit trees and ornamental plants. There is no treatment to these kinds of organisms as most are included in the EPPO quarantine list. Over the years, biomolecular techniques have been developed to detect and identify phytoplasmas, and the most important is nested-PCR for detection complemented with RFLP for identification and recently real-time PCR. Fruit tree phytoplasmas are widespread, mainly in Europe, and cause severe fruit production losses. This study concerned the apple proliferation group phytoplasmas that include three major phytoplasmas: ‘*Candidatus Phytoplasma mali*’, ‘*Ca. P. pyri*’ and ‘*Ca. P. prunorum*’, associated with apple proliferation (AP) and pear decline (PD) diseases in pome fruit trees and European stone fruit yellows (ESFY) in stone fruit trees. These phytoplasmas are vectored by psyllids *Cacopsylla picta*, *C. pyri* and *C. pruni*, respectively. Until now, has been no evidence of any of these diseases in Portugal, nor of their vectors, except for *C. pyri*, the only one reported until now in Portuguese pear orchards. Portuguese pear cv. Rocha is widely cultivated in Portugal, and is very important icon to national agriculture. This cultivar is exported worldwide, representing a major income in Portuguese economy. Due to its importance, it is fundamental to control all diseases infecting pear trees. As *C. pyri* is very abundant in Portuguese pear orchards and cause severe damages, it is important to know if the insect or the trees are infected with PD. As there are no previous records of the disease in Portugal, the main goal of this study was to assess the presence of the disease in order to prevent and control future spread. As the other two vectors have never been found in Portugal, one of the goals of this study was to look for them in apple orchards (where *C. picta* feeds) and prunus (peach, apricot, and cherry) orchards (where *C. pruni* feeds). The search was not successful so testing for AP and ESFY phytoplasmas was limited to vegetal samples, all negative for Portuguese orchards. Some of the captured insects were found to be positive for the PD phytoplasma, as well as some pear tree samples. The positive results, obtained by nested-PCR were confirmed by RFLP, nucleotide sequencing and PD specific real-time PCR assays. Work on this disease is now in progress.

## ENRICHMENT OF PHYTOPLASMA DNA BY SELECTED OLIGONUCLEOTIDES AND PHI29 POLYMERASE AMPLIFICATION

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Phytoplasma have resisted all attempts at cell-free cultivation to date, which hampers genome research. Elaborate and material intensive approaches are used to enrich the phytoplasma DNA. We present an amplification-based approach to obtain phytoplasma DNA from a few grams of plant tissue for downstream applications such as genomic draft sequencing. Total DNA was extracted by CTAB extraction from tobacco and parsley infected by stolbur strains 284/09 and 231/09, respectively. For enrichment, genomic DNA was amplified using oligonucleotides deduced from the four published complete phytoplasma genomes, random hexamers and Phi29 polymerase. Twenty-eight oligonucleotides were selected by frequency and distribution in the complete genomes. In additional experiments, the application of phytoplasma-specific primers P1/P7 was evaluated. *De novo* assemblies of short-reads with a length of 36 bases were generated. BLASTX against NCBI's NRPROT database using contigs with a minimum length of at least 300 bp was analysed with MEGAN and taxonomical assignment of the contigs performed. Enrichment was measured by illumina sequencing using a synthesis approach. Up to a fifteen-fold increase of the obtained phytoplasma draft sequence resulted from using determined oligonucleotides. Individual assemblies of short single-reads resulted in an average contig length of 1.3 kb for strain 231/09 and 2.5 kb for strain 284/09 and a total contig length of >474 kb and >498 kb, respectively. Preliminary results indicate only weak phytoplasma enrichment in the amplification experiments supplemented with P1 and P7 oligonucleotides. Combining the reads of the individual experi-

ments resulted in a draft sequence of >516kb for strain 231/09 and >557 kb for strain 284/09. Sequencing and annotation results highlight the potential of this strategy for uncharacterized phytoplasma genomes in particular. Cost-saving short-read sequencing has been shown to generate efficient draft sequences from these templates.

**GENETIC DIVERSITY, MEMBRANE TOPOLOGY, AND  
RELATIONSHIP TO VIRULENCE OF THE AAA+ ATPASES  
AND HFLB PROTEASES OF  
'*CANDIDATUS PHYTOPLASMA MALI*'**

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One of the economically most important phytoplasma pathogens in Europe is '*Candidatus* *Phytoplasma mali*', the agent associated with apple proliferation disease. Like other phytoplasmas, '*Ca. P. mali*' resides in plants in the sieve elements of the conducting phloem tissue where it can induce severe histopathological aberrations including the deposition of pathological callose and sieve tube necrosis. These symptoms lead to the accumulation of starch in the aerial parts of the trees and depletion of starch in the roots and may severely affect the performance of the trees. In more recent studies, the accumulation of hydrogen peroxide and phloem protein and the upregulation of genes involved in synthesis of these compounds have also been identified. In addition to this data on possible pathogenicity factors, a phylogenetic comparison of sequences of one of the *hflB* genes (AP464) annotated in the '*Ca. P. mali*' chromosome revealed that mild and severe strains cluster separately, according to their virulence. *HflB* (synonym *ftsH*) genes encode membrane-associated ATP- and Zn<sup>2+</sup>-depending proteases that are conserved among bacteria and degrade misassembled and thus contribute to the quality maintenance of proteins in the membrane and the cytoplasm. HflBs consist of ATPase domains and a protease module. The high copy numbers of *hflB* genes in '*Ca. P. mali*' and other phytoplasmas is unusual in prokaryotes and seems to indicate their importance for these plant pathogens. To further investigate the possible role of HflB proteins in the virulence of '*Ca. P. mali*', we PCR-amplified and sequenced all genes annotated as *hflB* in the chromosome of strain AT from a representative number of mild and severe strains. Analysis of deduced amino acid sequences revealed that seven of the annotated HflBs lack the protease module and should thus be classified as AAA+ ATPases (ATPases Associated with various cellular Activities). Furthermore, several predictors indicated that the enzymatically relevant C-termini of most of the

AAA+ ATPases and about half of the HflB proteases are facing the extracellular space and may thus be involved in the impairment of sieve element function. In addition it was found that full-length genes from the only cytoplasmatic ATPase (AP11) can only be amplified from mild and moderately virulent strains whereas severe strains yielded truncated genes only. Degeneration was also observed in one of the cytoplasmatic proteases (AP454) from which only a fragment could be amplified from all strains. In contrast, amplification of all AAA+ proteins facing the extracellular space resulted in full-length genes. In addition, phylogenetic analysis showed that the amino acid sequences of mild and severe strains of most of the proteins examined cluster far apart, mostly associated with the presence of virulence-related amino acid markers. In conclusion, the data reported here support previous indications that the AAA+ ATPases and HflB proteases of '*Ca. P. mali*' appear to be involved in pathogenicity.

## **TRANSCRIPTOME ANALYSIS OF *VITIS VINIFERA* cv. CHARDONNAY INFECTED WITH GLRaV-3 AND ASTER YELLOWS PHYTOPLASMA IN A SOUTH AFRICAN VINEYARD**

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Grapevine is one of the most important fruit crops grown in South Africa. Fruit productivity and quality can be greatly affected by diseases caused by various pathogens. Grapevine leafroll disease is prevalent in the grapevine production areas of South Africa. More recently, vineyards with aster yellows (AY) phytoplasma infection were reported in parts of the Western Cape winelands of South Africa and AY is becoming a threat to the grapevine industry. Using next-generation sequencing, we investigated the effects of *Grapevine leafroll associated virus-3* (GLRaV-3), the predominant virus associated with leafroll disease in South Africa and aster yellows phytoplasma on the transcriptome of *Vitis vinifera* cv. Chardonnay plants grown in the field. Young shoots were collected from a 6 year-old vineyard from the Western Cape region infected with GLRaV-3 and AY. The samples were tested for the presence of common grapevine viruses in South Africa, GLRaV-3 and aster yellows phytoplasma. Twelve samples were selected for RNA sequencing: three samples negative for common viruses, GLRaV-3 and AY; three positive for GLRaV-3; three for aster yellows and the remaining three were positive for both GLRaV-3 and AY. Total RNA was extracted from these samples and sequenced using the Illumina Hiseq 2000 platform. The infected samples were compared to non-infected samples. Preliminary data showed that more genes are differentially expressed in the mixed infected plants (GLRaV-3 and aster yellow) compared to singly infected plants. The significance of the data will be discussed. To our knowledge, this is the first report investigating the effects of mixed infection between a grapevine virus and phytoplasma from field samples.

## SUMMARY OF QUESTIONNAIRE DATA FOR THE PRESENCE OF PHYTOPLASMA DISEASES AND THEIR PUTATIVE VECTORS THROUGH EUROPE AND MIDDLE EAST

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Phytoplasmas occur in hundreds of commercial and wild host plants causing different diseases. In nature they are mainly spread by insects of the families *Cicadellidae* (leafhoppers), *Cixiidae*, *Delphacidae* and *Derbidae* (plant-hoppers), and *Psyllidae* (psyllids). With the development of diagnostic techniques the number of new phytoplasma diseases described worldwide has markedly increased. However, there are still no effective control measures able to reduce the spread and the damage of phytoplasma diseases in many crops, mainly due to the lack of information on their epidemiology. Therefore, one of the main tasks of the COST action FA0807 - WG2, which concerns phytoplasma epidemiology and vector ecology, is the establishment of a phytoplasma and vector monitoring system throughout Europe. For this purpose, a questionnaire on phytoplasma diseases and their putative vectors throughout European regions has been drafted and distributed to all members of the COST action FA0807. The obtained data have been used to create a database on the presence of phytoplasma diseases and their putative insect vectors in several European and Middle East regions. The database, consisting of 8 maps and detailed tables from 28 countries is available at the following web-site: <http://www.costphytoplasma.eu/InsectVectors.htm>. The maps summarize the spread of the five major phytoplasma-caused diseases (Apple proliferation, European stone fruit yellows, Pear decline, Bois noir and Flavescence dorée) and of their confirmed and putative vectors. Only the insect species that were proven to transmit phytoplasma to the host plant in that country by transmission trials were considered confirmed vectors, while species that are vector in another country or just tested positive by PCR were considered putative vectors. In the tables, detailed information on phytoplasma presence (strain/s, incidence, host/s symptoms, detection methods) as well as on the confirmed and putative vectors (population density, infection rate, phytoplasma detection tools, host plant/s, collection method/s, identification method/s) is provided for each country. All the data are supported by bibliographic references.

## PSYLLID VECTORS OF THE AP GROUP (16SrX) PHYTOPLASMAS IN TURKEY

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A survey was conducted in 2010 and 2011 in order to determine the presence of known vectors of apple proliferation group phytoplasmas (16SrX), namely ‘*Candidatus* Phytoplasma prunorum’, ‘*Ca. P. mali*’ and ‘*Ca. P. pyri*’, in different plants hosts (wild plums, conifers, and/or orchard trees). *C. pruni* individuals were collected from *Prunus spinosa*, some other *Prunus* spp. and *Abies* spp. from various provinces of Turkey. Molecular typing based on COI genes and an ITS region indicated that all individuals were from the species B. ‘*Ca. P. prunorum*’ infection rate in overwintered adults of *C. pruni* was estimated as 4.70%. Phytoplasma infection rate in new generation adults of *C. pruni* collected only the Mersin province was estimated as 4.64%. Psyllids, collected from wild and cultured forms of pome fruit trees, were identified as *C. picta*, *C. melanoneura*, *C. affinis*, *C. crataegi*, *C. pyrisuga*, *C. pyri* and *C. pyricola* according to their morphological characters. The most common psyllid among the collected ones, *C. picta*, was infected with ‘*Ca. P. mali*’ with a ratio of 4.36%, but one sample was infected by ‘*Ca. P. prunorum*’. *C. crataegi* individuals collected from hawthorn were also infected by ‘*Ca. P. mali*’ (2.90%). *C. melanoneura* and *C. affinis* were infected by ‘*Ca. P. pyri*’ with respectively a ratio of 3.63% and 2.27%, respectively, and never by ‘*Ca. P. mali*’. The psyllid *C. pyri* were found positive for ‘*Ca. P. pyri*’ (2.75%). The individuals of the species *C. pyrisuga* and *C. pyricola* were negative for phytoplasma. These preliminary data indicated that, *C. pruni*, the main vector of ‘*Ca. P. prunorum*’ is prevalent in Turkey. The main vector of ‘*Ca. P. pyri*’ was *C. pyri*, and *C. picta* might be the main vector of ‘*Ca. P. mali*’ Experimental transmission assays using these vectors are in progress.

## DEGREE OF SPECIFICITY AMONG THE PSYLLID-PHYTOPLASMA INTERACTIONS AND CONSEQUENCES IN EPIDEMIOLOGY

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Psyllid vectors often show close association with the pathogens they transmit. In particular, the eight known vectors of the genus *Cacopsylla* transmit only one phytoplasma species, and in contrast a single phytoplasma species may be transmitted by only one or no more than two psyllid species. When two species are described they are generally very closely related from a taxonomic point of view, such as for the vectors of 'Candidatus Phytoplasma pyri'. Recent results confirmed this degree of specificity of the vector-psyllid interaction (*C. pruni* species complex) or suggested that this could be even more specific (different populations of the same species would be able to have a distinct transmission efficiency against 'Ca. P. mali'). On the other hand, other reports suggest that several species living on the same host plant may transmit the same phytoplasma ('Ca. P. mali' and the psyllids on hawthorn). We discuss why these new results have important consequences for the investigation and the risk assessment of the role of a specific psyllid species in the spread of a phytoplasma disease.

## PHYTOPLASMAS ASSOCIATED WITH APRICOT CHLOROTIC LEAFROLL DISEASE

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### Abstract

Samples from apricot trees showing typical chlorotic leafroll symptoms were collected in June/July 2010 in Serbia and Italy. Phytoplasma detection was achieved by polymerase chain reaction and restriction fragment length polymorphism analyses on amplicons obtained after nested-PCR assays. 16SrX-B, 16SrXII and 16SrI phytoplasmas were identified in single or mixed infection in the Serbian samples, while 16SrX-B, 16SrXII and 16SrIX-C were detected in single or mixed infection in the Italian samples. This is the first report of the presence in symptomatic apricot of different phytoplasmas from ESFY, in particular the 16SrIX-C phytoplasma is reported for the first time in this species. Work to verify the epidemic relevance of these phytoplasmas in the apricot chlorotic leafroll disease is in progress.

### Introduction

An apricot decline of a possible infectious origin reported in 1934 (Goidanich, 1934), and later a similar disease described as apricot chlorotic leaf roll (ACLR) (Morvan, 1977) or apoplexy in some cases, was described in several European countries. The affected trees show, initially on some branches, symptoms of deficiency in water and nutrient supply which are expressed as rolling and chlorosis, followed by early reddening of leaves, metabolic disorders leading to sudden dieback during the early growing season. These phenomena are followed by the sudden death of whole branches or the entire crown. Electron or fluorescence microscopy enabled phytoplasmas in sieve tubes to be detected and transmission experiments to other stone fruit and indicator plants were successfully carried out thus demonstrating phytoplasma association with this disease (Morvan, 1977; Goidanich *et al.*, 1980; Giunchedi *et al.*, 1982).

An increasing presence of chlorotic leaf roll disease in apricot (*Prunus armeniaca*) has been observed in commercial orchards in several European regions in the last thirty years (Desvignes and Cornaggia, 1982; Dosba *et al.*, 1991; Bertaccini *et al.*, 1993; Pastore *et al.*, 1999; Laimer *et al.*, 2001; Torres *et al.*, 2004; Duduk *et al.*, 2008). Apricot trees are affected by apricot chlorotic leafroll disease in all major cultivation areas in Europe: the disease kills the plants in a few years and it is widely



Fig. 1 - Branches (on the left) and plants of apricot showing ACLR symptoms and apoplexy respectively in the Serbian orchards.

associated with European stone fruit yellows (ESFY) ‘*Candidatus Phytoplasma prunorum*’ belonging to 16SrX-B subgroup (Lee *et al.*, 1998; Seemüller and Schneider, 2004). A survey was carried out on a restricted number of plants in Italy and Serbia to confirm phytoplasma presence in apricot orchards where the symptomatology was quite severe and present in a non epidemic situation.

### Materials and Methods

Samples from apricot trees showing chlorotic leafroll symptoms (Fig. 1) very often followed by apoplexy were collected in June/July 2010 in Serbia and Italy in scattered plants from 7 and 8 year-old orchards. Three apricot varieties were tested in Italy: Cafona, Portici, and San Francesco and one unknown variety was tested in Serbia. Leaf and young branch samples from 10 symptomatic and two asymptomatic plants were collected and leaf midribs and phloem scrapes were employed for nucleic acid extraction carried out following reported procedures (Prince *et al.*, 1993). Phytoplasma detection was then achieved by PCR assays amplifying 20 ng of template DNA with primers P1/P7 (Deng and Hiruki, 1991; Schneider *et al.*, 1995) followed by primers F1/B6 (Duduk *et al.*, 2004), R16F2n/R2 (Gundersen and Lee, 1996) or PA2f/r (Heinrich *et al.*, 2001) in nested PCR test carried out on amplicons diluted 1:30 with sterile distilled water as template. Further nested-PCR assays were performed using the group specific primers R16(I)F1/R1 (Lee *et al.*, 1994) and R16(X) F1/R1 (Lee *et al.*, 1995). A tube with reaction mixture devoid of DNA template was included as a negative control in each PCR reaction. All PCR amplifications were performed following published parameters (Schaff *et al.*, 1992). PCR products were

electrophoresed in 1.0% agarose gel, the gel was stained with ethidium bromide and visualised under UV light. RFLP analyses with *TruII*, *RsaI*, *HinfI*, *HhaI*, and *HpaII* were carried out on F1/B6, R16F2/R2 amplicons. Digestion was carried out for 16-30 hrs at 37 or 65°C according to the manufacturer's instructions for each enzyme. The products were separated by electrophoresis in 7% polyacrylamide gel, and visualised under a UV transilluminator.

### Results and Discussion

Only a nested-PCR assay led to the detection of bands in all symptomatic apricot samples tested and the use of phytoplasma group specific primers showed the presence of mixed phytoplasma infection in some of the samples (Tab.1).

Tab. 1 - Results of phytoplasma identification in apricot samples from plants showing apoplexy symptoms in Italy and in Serbia.

Apricot varieties or number	Direct PCR with P1/P7 followed by nested PCR primers			Identified phytoplasmas
	R16F2/R2	R16(I)F1/R1	R16(X)F1/R1	
Italy				
Portici Vecchio	-	-	+	16SrX-B
Cafona	+	-	+	16SrX-B + 16SrlX-C
Portici	+	-	+	16SrX-B
San Francesco	+	+	-	16SrXII-A
Serbia				
A20/10	+	+	+	16Srl+16SrXII-A+16SrX-B
A23/10	+	+	+	16SrX-B+16SrXII-A
A24/10	-	+	-	16SrXII-A
A26/10	+	-	+	16SrX-B

Collective RFLP analyses with *TruII*, *RsaI*, *HinfI*, *HhaI*, and *HpaII* on R16F2n/R2 amplicons showed the presence of 16SrX-B ('*Candidatus* Phytoplasma prunorum', ESFY) phytoplasmas in six samples, three from the Italian orchard and three from the Serbian one. However only one sample from Italy and from Serbia showed single ESFY infection. In the other two Serbian apricot samples mixed infections with 16SrXII (stolbur) and with stolbur and 16Srl (aster yellows) phytoplasmas were identified. In two of the remaining samples from each country stolbur phytoplasmas were identified in single infection, while in the last sample from Italy the 16SrX-B phytoplasma was in mixed infection with 16SrlX-C phytoplasmas (Fig. 2). Only the use of nested PCR with PA2 primers on P1/P7 was able to detect 16SrXII phytoplasmas in the last sample from Serbia (data not shown). In all the experiments carried out the two asymptomatic plants were negative.

This is the first report of the presence in symptomatic apricot of phytoplasmas different from ESFY, in particular aster yellows, stolbur), and 16SrlX-C ('*Candidatus*

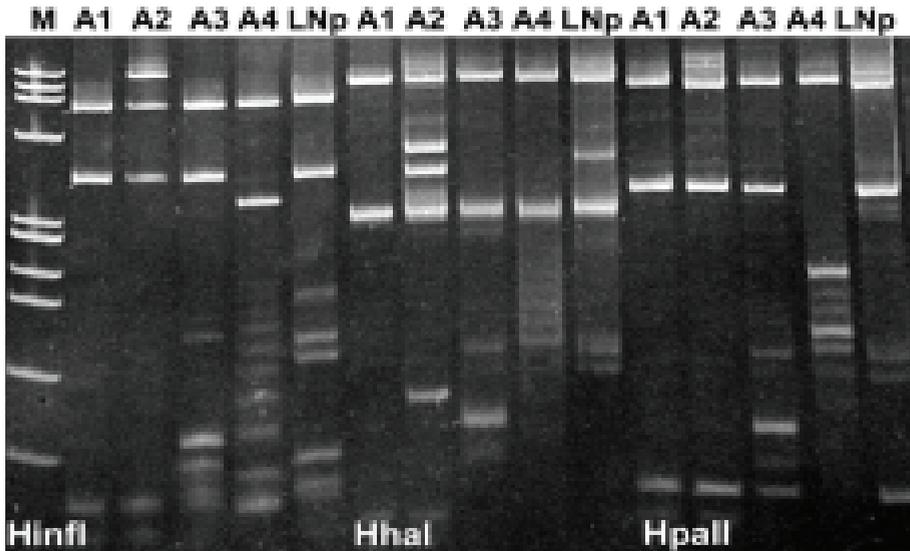


Fig. 2 - Results of RFLP analyses with restriction enzymes listed at the bottom of the figure on amplicons obtained from apricot samples from the Italian orchards samples (A1-A4) after amplification with primers R16F2/R2; LNp, periwinkle infected with European stone fruit yellows phytoplasma (ESFY, 16SrX-B). M, marker  $\Phi$ X174 HaeIII digested, fragment sizes (from top to bottom): 1,353; 1,078; 872; 603; 310; 281; 271; 234; 194; 118, and 72.

phytoplasma phoenicium'). These phytoplasmas were detected mainly in mixed infection and in plants with apoplexy symptomatology. In the Italian orchards a small scale survey to verify presence of phytoplasma vectors was also carried out and a few specimens of *Cacopsylla pruni*, *C. pulchella* and *Fieberiella florii* were collected, identified and tested for phytoplasma presence and only one specimen of *C. pulchella* was found carrying 16SrX-B phytoplasmas. There is no experimental evidence of ESFY phytoplasma transmission by this insect, however *Cacopsylla pruni* is the reported vector (Carraro *et al.*, 1998). No insect search was performed in the Serbian orchard. Work to further verify the association of this syndrome with the phytoplasma presence and to determine the epidemic spreading of this disease in Italy and in Serbia is in progress.

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**ALMOND WITCHES' BROOM PHYTOPLASMA,  
OFFICIALLY DECLARED AS A REGULATED PEST IN  
LEBANON**

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Surveys conducted during 2009/10 showed that almond witches' broom phytoplasma (AlmWB) disease, incited by '*Candidatus* Phytoplasma phoenicium', was widespread in Lebanon and was detected in 16 out of 26 districts. Almond, peach and nectarine were severely affected. The north of Lebanon was considered as the epidemic center from where the disease had spread to the other regions. Due to the devastating losses already incurred and in order to reduce further losses, HE the Minister of Agriculture held a press conference on January 17, 2011 during which he officially declared AlmWB as a regulated pest in Lebanon and that a national plan would implemented for the integrated management of the disease. Farmers will be encouraged to eliminate infected trees in all areas and will be compensated by providing free seedlings of replacement crops. The major stone fruit cropping regions in Bekaa (West Bekaa and Rachayya) will be given priority and the extension service will actively help in the eradication process. The field activities started on March 2012 with financial support from the Italian government. Updates on the actions taken will be presented during the meeting.

## MONITORING OF THE DISTRIBUTION OF FRUIT TREE PHYTOPLASMAS IN BULGARIA FROM 2007 UNTIL 2011

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### Abstract

Apple proliferation (AP), European stone fruit yellows (ESFY) and Pear decline are economically highly important disorders in many fruit tree growing regions in Europe and also in Bulgaria. A survey was conducted from August to November between 2007 and 2011 in selected fruit tree orchards in 28 districts in Bulgaria. The results of this five year survey show that '*Candidatus Phytoplasma mali*' causing apple proliferation (AP), '*Candidatus Phytoplasma prunorum*', the agent of European stone fruit yellows (ESFY) and '*Candidatus Phytoplasma pyri*', the agent of Pear decline (PD) are present in Bulgaria. Phytoplasma-infected plants were found in 10 of the 28 investigated regions.

### Introduction

Apple proliferation (AP) caused by '*Candidatus Phytoplasma mali*', European stone fruit yellows (ESFY) caused by '*Candidatus Phytoplasma prunorum*' and Pear decline caused by '*Candidatus Phytoplasma pyri*' (Seemüller and Schneider, 2004) are of the most important diseases of fruit trees in Bulgaria, after *Plum pox virus*, and until now the existence and spread of fruit tree phytoplasmas and their vectors, have not been researched in detail.

ESFY and PD were first detected in Bulgaria near the town of Plovdiv (Topchiiska *et al.*, 2000). AP has been reported to occur in Bulgaria for a long time.

The phytoplasmas' research and control are part of the official fruit trees monitoring program of the National Service on Plant Protection (now Bulgarian Food Safety Agency).

A survey was conducted from August to November between 2007 and 2011 in selected fruit tree orchards in all 28 districts in Bulgaria. The controlled area, covered by this monitoring program extends to 2000 ha per year. Around 1500 samples have been tested for phytoplasma infection annually, and the number of samples has varied from region to region (annual reports of 'Official fruit trees monitoring program' 2007-2011).

The aim of the present work was to draw a picture of the occurrence and spread of all three fruit tree phytoplasmas in Bulgaria.

### Materials and Methods

During the described period plant material was derived from symptomatic and non-symptomatic trees and was sampled in late autumn when the phytoplasma concentration in the aerial tree parts is highest. The main priority was to collect samples from plant material originating from fruit tree nurseries and mother stock samples. All samples were analyzed by PCR for phytoplasma infection in the Central Laboratory for Plant Quarantine. DNA was extracted with a CTAB-based protocol as described by Maixner *et al* (1995). PCR amplification of phytoplasma DNA was tested by nested PCR, followed by RFLP analysis using restriction enzymes *RsaI* (AfaI) and *AluI* (Amersham Biosciences, USA).

### Results and Conclusion

During the described period phytoplasma-infected plants were found in 10 of the 28 investigated regions. (Figure 1.)

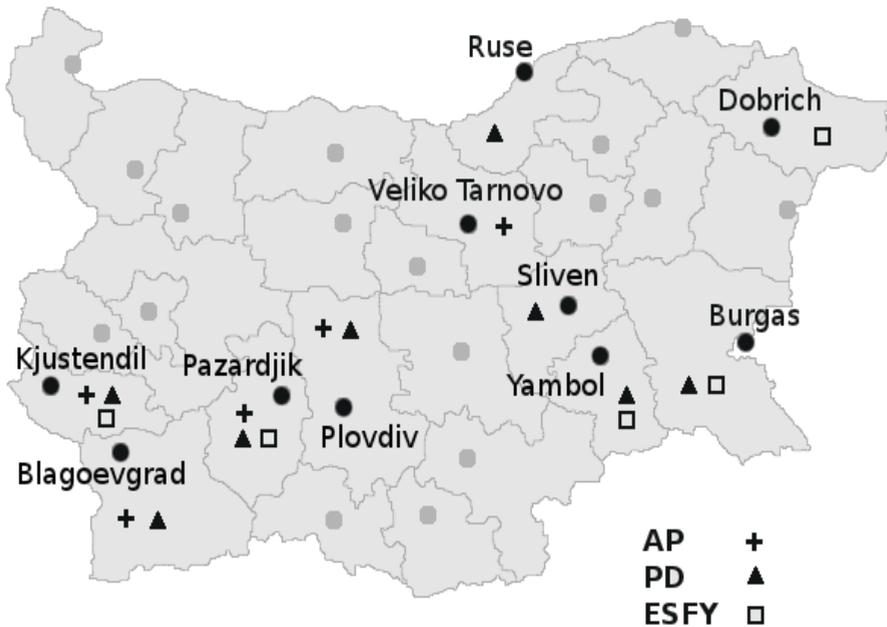


Fig. 1 - The distribution of AP, ESFY and PD in Bulgaria.

‘*Candidatus Phytoplasma mali*’ the agent of Apple proliferation (AP) and ‘*Candidatus Phytoplasma prunorum*’, the agent of European stone fruit yellows (ESFY) were found in samples in five different regions. Pear samples from 7 different regions in Bulgaria were found to be infected with ‘*Candidatus Phytoplasma pyri*’, the agent of Pear decline (PD).

The incidence of Apple proliferation in 2009 and 2010 was lower than in 2007 and 2008, because of the applied phytosanitary measures. As a result, in 2011 no cases of AP infection were found. ESFY infection was found in 2 samples in 2009, in 5 samples in 2010, and in 5 samples in 2011, PD infection was detected in all 5 years, with a minimum value of 2 samples in 2009 and maximum of 6 samples in 2010.

The infected plants were destroyed through incineration. Related plants were also monitored additionally, and in case of phytoplasma infection were destroyed. Any transfer of plants from infected fields has been prohibited. Insecticide treatments have been applied against putative phytoplasma vectors in these areas. Phytosanitary prevention measures should be taken for ESFY and especially for PD to minimize the spread of infection.

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## **INDUCTION OF DEFENSE GENES IN APPLE LEAF TISSUES BY AN ENDOPHYTIC STRAIN OF *EPICOCUM NIGRUM* AND ITS POSSIBLE USE IN THE CONTROL OF THE APPLE PROLIFERATION DISEASE**

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Endophytes are micro-organisms living inside host plants without causing disease symptoms or visible injury. The ecological role of these organisms is still not well determined but most exhibit positive effects for the host plants by promoting growth, improving resistance to multiple stresses and protecting them against pathogens and insects. Apple proliferation (AP), associated with ‘*Candidatus Phytoplasma mali*’ (‘*Ca. P. mali*’) is one of the most economically important disease affecting apple in Europe. The possibility of using endophytes as biocontrol agents or resistance-inducers against phytoplasmas has been recently reported. Musetti *et al.*, reported that an endophytic strain of *Epicoccum nigrum*, inoculated in *Catharanthus roseus*, experimentally infected with ‘*Ca. P. mali*’, reduced symptom severity and phytoplasma titre inside the plant tissues, inducing ultrastructural modifications both to the phytoplasma and to the host. Starting from these promising results and aimed at assessing the possibility of using *E. nigrum* in the AP control an experiment was carried out using the natural host of ‘*Ca. P. mali*’, *Malus domestica*. Four groups of young apple trees (10 individuals per group) were set up: only *E. nigrum* was inoculated, E<sup>+</sup> AP<sup>-</sup>; *E. nigrum* inoculated then infected with ‘*Ca. P. mali*’, E<sup>+</sup> AP<sup>+</sup>; only ‘*Ca. P. mali*’ infected, E<sup>-</sup> AP<sup>+</sup>; control plants E<sup>-</sup> AP<sup>-</sup>. AP symptom expression, plant and phytoplasma ultrastructural modifications as well as phytoplasma titre in the different groups of plant were evaluated, together with the expression of some defence-related genes. In this work, we investigated the expression of apple genes coding for three pathogenesis-related (PR) proteins, PR 1, PR 2, PR 5 and for three jasmonate (JA)-pathway marker enzymes, Allene Oxide Synthase 2 (AOS 2); 12-Oxyphytyldienoate reductase 3 (12-OPR 3); JA-inducible Proteinase Inhibitor II (PI II), comparing their relative expression levels in apple plants that were or were not inoculated by *E. nigrum* (E<sup>+</sup>AP<sup>-</sup> and E<sup>-</sup>AP<sup>-</sup> plant groups). Expression profiling of the selected genes revealed a different expression pattern according to the two different plant groups. In particular, PR1 and PR5 were significantly induced (8 and 4 folds respectively) in E<sup>+</sup>AP<sup>-</sup> plants 3 days after endophyte inoculation (dai) compared to the non-inoculated controls (E<sup>-</sup>AP<sup>-</sup>). The expression level of the induced genes tends to return comparable results to the control two weeks and two months after endophyte inoculation. On the other hand variations in the expression level of the

genes encoding the three JA-pathway marker enzymes at 3 dai do not seem to be considerable in E<sup>+</sup>AP<sup>-</sup> leaf tissues compared to the control. From these preliminary results, we can hypothesize that *E. nigrum* inoculation can induce defense responses in apple related to the salicylic acid pathway and PR protein gene expression, inhibiting, on the other hand, a JA-related defense pathway. Further analyses will be carried out aimed at understanding the mechanism(s) by which *E. nigrum* interacts with apple plants and to assess the real possibility of its usefulness in AP disease control.

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